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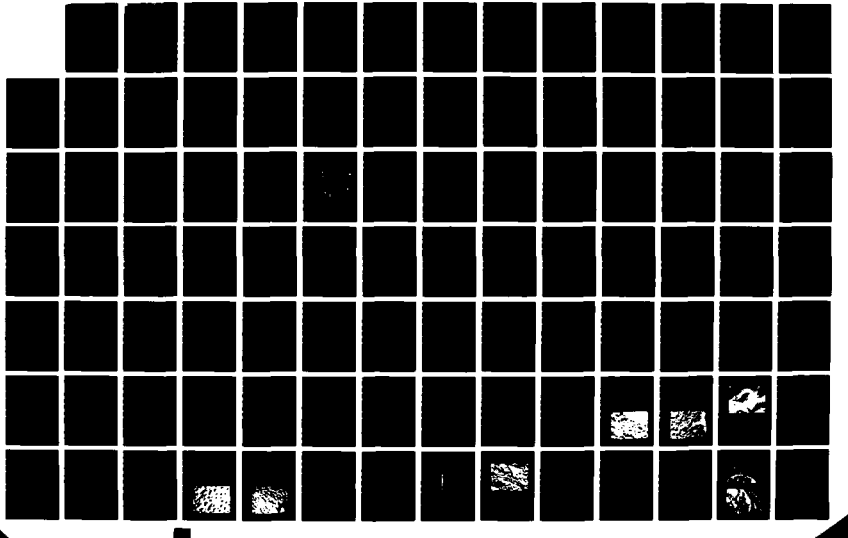

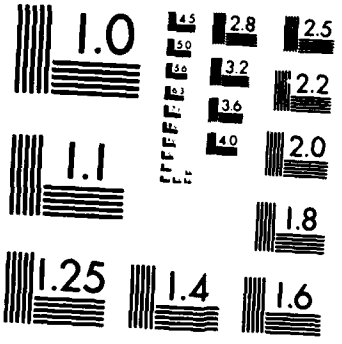

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ABSTRACT

Previous studies on mice with severe nephrogenic diabetes insipidus (NDI) had suggested that their urinary concentrating defect was due to an inability to increase the water permeability of the collecting duct system. Other studies, utilizing vasopressin-sensitive amphibian epithelia, have reported the appearance of intramembranous particle (IMP) clusters in luminal membranes, possibly arising from cytoplasmic vesicles, during states of increased water permeability. The present study was designed to help characterize at the cellular level, the urinary concentrating defect of NDI mice. → next page

Freeze fracture and thin section electron microscopy were used to examine principal cells from inner medullary collecting ducts of several strains of mice for the presence of IMP clusters in luminal membranes and for cytoplasmic vesicles near the luminal surface. The strains included: DI +/+ Severe mice (florid DI), DI +/+ Nonsevere mice (moderate deficiency of urinary concentration), VII +/+ Normal mice (controls), and CBA Os/+ mice (moderate deficiency of urinary concentration in association with undersized kidneys).

Urinary osmolality (U_{osm}), percentage of cells with IMP clusters, and number of uncoated cytoplasmic vesicles within one micrometer of the luminal surface were measured and are summarized below (mean±SEM):

Mouse Strain	U_{osm} mOsm/kg H_2O	Positive Cells (%)	Cytoplasmic Vesicles per micrometer
VII +/+ Normal	2234±190 ¶	52±5 Φ	1.47±.57 ∇
DI +/+ Severe	203±43 ¶	0.0 Φ	0.49±.26 ∇
DI +/+ Nonsevere	1133±86 ¶	33±4 Φ	1.21±.57 ∇
CBA Os/+	2043±95	84±6 Φ	not measured

¶ Φ ∇ = statistically different, P<0.05.

There was a strong correlation between IMP cluster frequency and U_{osm}, and between IMP cluster frequency and numbers of cytoplasmic vesicles.

The present study provides evidence that the defect in urinary concentrating ability found in NDI mice, both Severe and Nonsevere, may be partly due to a lack of IMP clusters, which probably refelects deficient vasopressin-induced water permeability of the collecting duct system. This defect is probably not identical to the defect(s) previously observed in CBA Os/+ mice.

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Intramembranous Particle Clusters and Cytoplasmic Vesicles in Mice with Nephrogenic Defects of Urinary Concentration

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With the exception of the urinary osmolality in CBA Os/+, all variables were statistically different from each other. The IMP clusters of CBA Os/+ mice contained significantly more IMPs than did those of VII +/+ Normal or DI +/+ Nonsevere mice. There was also a strong correlation between IMP cluster frequency and U_{osm}, and between IMP cluster frequency and numbers of cytoplasmic vesicles.

The present study provides evidence that the defect in urinary concentrating ability found in NDI mice, both Severe and Nonsevere, may be partly due to a lack of IMP clusters, which probably refelects deficient vasopressin-induced water permeability of the collecting duct system. This defect is probably not identical to the defect(s) previously observed in CBA Os/+ mice. In this study, the CBA Os/+ mice had a mean U_{osm} measurably lower than VII +/+ Normal mice (not statistically different), despite having a greater proportion of cells with IMP clusters.

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GLOSSARY

AdC	adenylate cyclase
aggregates	a term used interchangeably with IMP cluster; groups of intramembranous particles, thought to mediate water permeability
aggrephores	cytoplasmic vesicles thought to contain preformed IMP clusters for inclusion into the membrane upon stimulus
antidiuretic hormone (ADH)	hormone released from posterior pituitary gland in response to water deficit
anuran	descriptive adjective for "frog-like" amphibians; includes toads
apical	a term used interchangeably with luminal to describe a portion of the cell's plasma membrane
basolateral	the side opposite the luminal side; in principal cells of mammalian collecting ducts this corresponds to the blood side of the cells
catalytic subunit (C)	portion of adenylate cyclase which upon activation promotes the conversion of ATP into cAMP
cAMP	cyclic-3',5'-adenosine monophosphate; sometimes referred to as a cellular second messenger
cluster	easily recognizable tight grouping of intramembranous particles, often seen in luminal membranes of cells responsive to neurohypophysial hormones
collecting duct system	connecting segment, initial collecting tubule, and cortical, outer medullary, and inner medullary collecting duct of the nephron
cortical	referring to that section of the kidney called the cortex; section ranges from outer surface to beginning of the medulla
corticopapillary	in the direction of cortex to papilla; often used to refer to osmotic gradient established in interstitium of kidney
DDAVP	1-desamino-8-D-arginine vasopressin; synthetic analogue of vasopressin
DI	diabetes insipidus

disequilibrium	in this study, a lack of osmotic equilibration between fluid in the collecting duct system and the surrounding papillary interstitium
E face	the inside surface of that half of the membrane lipid bilayer which was, prior to freeze fracture, the outside of the membrane
filipin	an antibiotic which complexes with sterols; used as a cytochemical probe for cholesterol and/or coated pits
G_i	inhibitory guanine nucleotide regulatory unit; part of adenylate cyclase
granules	in the toad urinary bladder, membrane-bound vesicles which contain darkly staining substances, as yet unidentified; not present in principal cells of mammalian collecting duct
G_s	stimulatory guanine nucleotide regulatory unit; part of adenylate cyclase
GTP	guanosine triphosphate
HRP	horseradish peroxidase; an electron-opaque substance used to identify endocytotic or exocytotic cellular events
IMP	intramembranous particle; grain-like protuberance observable in the leaflets of the phosopholipid bilayer of the plasma membrane during freeze fracture electron microscopy; thought to be embedded proteins
leaflet	term which refers to one half of the membrane lipid bilayer
L_p	transepithelial water flow (centimeters • second^{-1} • atmosphere^{-1})
luminal	that membrane of a bipolar epithelial cell, which faces the lumen; most often this is the side with variable transport properties
mA	milliamperes; electrical current
medullary	referring to that section of the kidney called the medulla; section ranges from the innner boundary of the cortex to calyces and renal pelvis; divided into inner and outer portions
MIX	1-methyl-3-isobutyl xanthine; a specific inhibitor of cAMP phosphodiesterase activity
mOsm	milliosmols; one-thousandth of an osmol; computed by multiplying the millimolar concentration by the

	number of particles per mol obtained by ionization
NDI	nephrogenic diabetes insipidus
neurohypophysis	posterior pituitary gland
ng	nanogram
oligosyndactyly (Os)	a syndrome characterized by fused or missing digits; accompanied in certain strains of mice by small kidneys and chronic renal failure
osmolality	the number of osmotically active particles in a solution; computed by multiplying the molar concentration by the number of particles per mol obtained by ionization
$P_d(w)$	diffusional water permeability coefficient (centimeters•second^{-1})
PDIE	cAMP phosphodiesterase
P_f	osmotic water permeability coefficient (centimeters • second^{-1})
P face	the inside surface of that half of the membrane bilayer that, prior to freeze fracture, was touching the cytoplasm of the cell
r	correlation coefficient
replica	a platinum and carbon "impression or mold" of the vertical detail of the surface of a cell subjected to freeze fracture; the platinum, which is electron opaque, provides the image contrast
r_s	Spearman's Rho rank correlation coefficient
SEM	standard error of measurement; computed by dividing standard deviation by the square root of the number of samples
U_{osm}	urine osmolality; expressed as milliosmoles per kilogram of water (mOsm/kg H_2O)
V	volts; potential difference
vasopressin (VP)	alternative name for antidiuretic hormone (ADH)

1 INTRODUCTION

1.1 The Concentration of Urine

1.1.1 At the Nephron Level -- The production of hypertonic urine is a complex process that is the result of the interaction of two main mechanisms: (1) the buildup of an interstitial osmotic gradient, and (2) the exposure of tubular fluid to that gradient by cells with controllable water permeability. The cells of the collecting duct system are responsible for the latter; countercurrent multiplication (both passive and active) and the recycling of urea (among others) are primarily responsible for the former.

The operation of a countercurrent multiplication mechanism in the mammalian kidney was first suggested by Kuhn and Ryffel in 1942 (90), and subsequently clarified by numerous other investigators. It is a process which helps multiply the concentration of solutes in the medullary interstitium. During this process, increasing amounts of NaCl are deposited in the interstitium due to differential permeabilities to NaCl and water of portions of the descending and ascending limbs of the loops of Henle, and due to the hairpin-turn anatomical structure of the loops. In addition, urea may be either deposited or "washed out" passively, depending upon its tubular concentration and/or the rate of flow of the tubular fluid (169). The end result of these processes is an interstitial osmotic gradient with increasing concentration from cortex to medulla.

While the hairpin-turn arrangement of the loop of Henle helps to facilitate countercurrent multiplication of solutes, it is the turn back in the opposite direction, of distal tubules toward the collecting duct of each nephron and consequent passage of the collecting ducts through

the previously developed corticopapillary osmotic gradient, which can potentially expose the glomerular filtrate to a hypertonic, osmotic environment. Whether or not the filtrate is exposed to this gradient, and therefore whether or not appreciable free water is reabsorbed, resulting in a urine more concentrated than plasma, depends on the concentration of vasopressin in the blood [in the range of 10^{-12} to 10^{-11}M (66)]. The effect of vasopressin is to increase the water permeability of the cells comprising the collecting duct system. This action is mediated through the second messenger mechanism proposed by Sutherland (158). The ultimate result of increased water permeability in these cells is that the filtrate, which passes through these vasopressin-sensitive areas of the collecting duct system, may have free water reabsorbed osmotically due to the corticopapillary, interstitial osmotic gradient. This absorptive process helps to restore total body water levels to normal, and concomitantly to concentrate the urine. At least theoretically, with full water permeability (maximum response to vasopressin), the urine could be concentrated to the same osmolality as the interstitium of the collecting ducts through which it passes.

1.1.2 At the Cellular Level

1.1.2.1 Vasopressin Receptors -- The vasopressin in the blood results from neurohypophysial release in response to a decrease in total body water, most likely sensed by either osmoreceptors (170) or by blood volume receptors (169), or by a combination of both. The vasopressin which is released must then bind to appropriate cellular receptors in order to mediate physiological responses.

Two types of receptors, which bind vasopressin, called isoreceptors (72), have been identified in the mammalian kidney and have been designated V_1 and V_2 receptors (103). Although both receptors bind

vasopressin, the cellular biochemical responses evoked by the binding of this hormone to each of these receptors differs.

The close association now known to exist between vasopressin receptors (V_2) and adenylate cyclase, an enzyme which catalyzes the conversion of ATP to cyclic AMP (123), was reported by Bockaert et al (9). They found that the binding of vasopressin to its receptor activated adenylate cyclase. In addition, continued receptor occupancy was required for continued adenylate cyclase activity. Subsequently, other investigators reported that vasopressin stimulation caused an increase of intracellular cAMP in cells of mammalian collecting ducts (38,54,60). More recently, Dousa (40), Ausiello and Orloff (5), and Jard (72) have shown that it is the stimulation of the V_2 receptor specifically which results in increased intracellular cAMP. In addition, Eggena and Ma have reported that in toad bladder, the V_2 receptor can be downregulated by vasopressin itself (45).

In contrast, vasopressin stimulation of V_1 receptors does not directly affect the intracellular concentration of cAMP. In the mammalian kidney V_1 receptors have been found only in mesangial cells of the glomerulus and in medullary interstitial cells (41). Most data on cellular biochemical responses in the mammal that involve V_1 receptors have been gleaned from studies done on extrarenal tissues (41). Nevertheless, it is not unreasonable to expect that the V_1 responses observed in the other tissues would be similar, if not identical, to those which might be found in response to V_1 stimulation in the kidney.

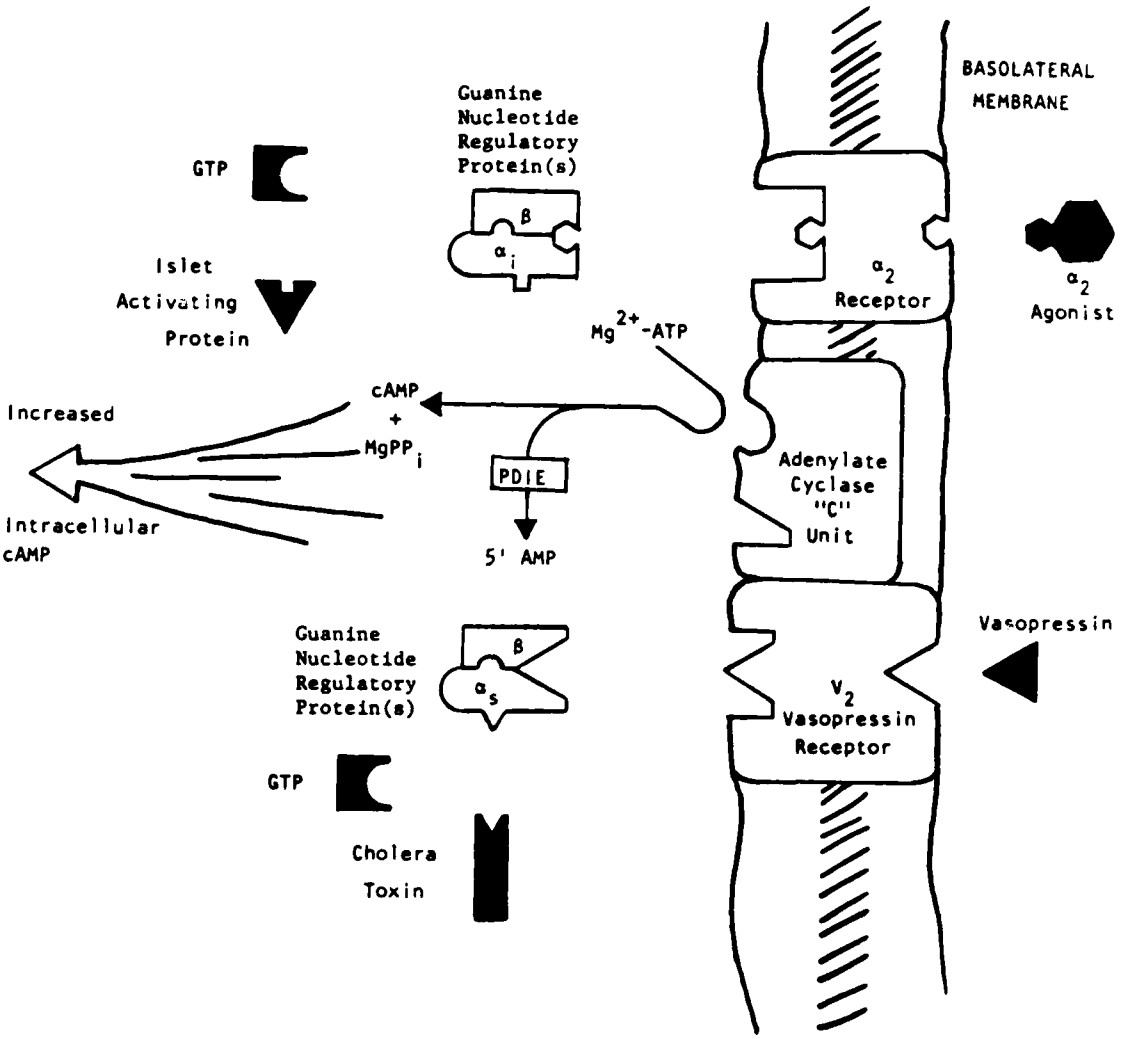
According to Berridge (8), stimulation of V_1 receptors with vasopressin leads to a rapid hydrolysis of phosphatidylinositol (PI), and an increased turnover of PI. Increased PI turnover is associated with intracellular Ca^{2+} fluxes, and the activation of phospholipase-A_2. Increased PI turnover also results in arachidonic acid release from the

plasma membrane, which can increase the synthesis of prostaglandins (8). Prostaglandins PGE_1 and PGE_2 have been shown in collecting tubules to have effects which counter cAMP-mediated responses to vasopressin stimulation. For example, in isolated, perfused, cortical collecting tubules of the rabbit, Grantham and Orloff (55) showed that PGE_1 inhibited the hydroosmotic response to vasopressin, but not the hydroosmotic response to exogenous cAMP, suggesting that PGE_1 interferes with the generation of cAMP. Similarly, Edwards et al (44) and Torikai and Kurokawa (162) reported that PGE_2 inhibited vasopressin-stimulated cAMP accumulation in individually dissected, cortical and papillary collecting tubules. Hence, these studies imply that the responses of V_1 and V_2 receptors to vasopressin may be interrelated. In fact, there are ample data (5,7,40,87) which suggest that PGE_2 (V_1 origin) may function as a negative feedback agent in the cAMP-mediated (V_2 origin) response of an increase in water transport by the collecting tubule (41).

Unfortunately, there are currently few data on the mechanism of the V_1 receptor in cells of the mammalian collecting duct. Therefore, the scope of the present study was limited to a consideration of the cellular response to vasopressin stimulation of the V_2 receptor. As noted above, occupation of this receptor results in the activation of adenylate cyclase (9), which catalyzes the conversion of ATP to cyclic AMP (Fig. 1).

1.1.2.2 Adenylate Cyclase -- The role of adenylate cyclase (AdC) in the conversion of ATP to cAMP has only recently come under close experimental scrutiny. High interest in this biochemical pathway, due to the ubiquitous nature of cAMP-mediated processes, has yielded a wealth of information in a short time. Adenylate cyclase is not a single, homogeneous enzyme. Rather, it is an enzyme system, composed of several

Figure 1. A model of the adenylate cyclase system. ($MgPP_i$ = magnesium pyrophosphate; GTP = guanosine triphosphate; PDIE = cAMP phosphodiesterase.



identifiable subunits. These subunits include a receptor (R), guanyl nucleotide regulatory proteins (G), and the catalytic unit (C)(51)[Fig. 1].

The receptor is regarded by some investigators to be part of adenylate cyclase. However, AdC systems in several different cell types

have shown analogous responses despite having receptors that are activated by totally different hormones. Actions of the V_2 receptor have already been discussed (see above). Therefore, further consideration of the receptor in the present study, as part of the AdC response, will assume that the transduction of the hormone "signal" which has been described for systems with other receptors, will prove to be essentially the same for the V_2 system. In this regard, both theoretical and experimental evidence suggest that the action of the receptor-hormone (or agonist) complex is to cause a dissociation of the guanine nucleotide stimulatory protein (G_s) heterodimer into its α and β subunits (51,144,161). To better understand the consequences of this action, a more detailed explanation of G_s as well as G_i, the inhibitory guanine nucleotide regulatory protein, is required.

The complicated roles that G_s and G_i play in the AdC response is reflected in the complexity of their structure. Both G_i and G_s are heterodimers, composed of two subunits, called somewhat confusedly, α and β. While the β subunits are apparently identical proteins of approximately 35000 Daltons (D), the α subunits are not (152). The $G_s\alpha$ subunit is approximately 45000 D (152) and is ADP-ribosylated by cholera toxin (activated to stimulate C)(107). The $G_i\alpha$ subunit is approximately 41000 D (152) and is ADP-ribosylated by the islet-activating protein fraction of pertussis (Bordetella pertussis) toxin (activated to inhibit C)(82-84). However, the α subunits do have in common a high affinity binding site for guanosine 5'-triphosphate, GTP. In this regard, Rodbell has shown that GTP is a mandatory cofactor for stimulation, or activation, of G_s (131). Theories on the operation of G_s and G_i within the overall AdC response are no less complex, but the models of Gilman (51) and Skorecki et al (144) furnish valuable insight into probable mechanisms.

As mentioned above, interaction of the hormone-receptor complex with G_s results in a conformational change (51), probably α- β dissociation. Additionally, the hormone-receptor complex promotes the binding of GTP to the α subunit (144). The α subunit complexes with the catalytic unit (C) and subsequently dissociates, leaving the activated C which promotes the conversion of substrate ATP into cAMP (144). Inhibition of the response can come from activation of the G_i subunit (i.e., pertussis toxin)(82-84) which results in dissociation of G_i into α and β subunits (51). While the activated $G_i\alpha$ subunit can cause inhibition itself, it is very weak, at least in vitro. It is more likely that inhibition is caused by the "extra" β subunits liberated by the dissociation, now available to combine with α subunits of G_s (51). Reconstitution studies using resolved subunits of G_s and G_i have demonstrated that the addition of β subunits to mixtures of G_s, G_i, and C prevents G_s and G_i from combining with C (10,153). At least in the model of Skorecki et al, which is based on radiation inactivation studies, inactivation can also be caused by GTPase activity, which changes GTP to GDP. The dephosphorylation results in the inactivation of the α_{GTP}-C complex and consequently, a smaller amount of active C. Additionally, in the Skorecki et al model, activated C undergoes inactivation over time, which diminishes its ability to enzymatically change ATP to cAMP (144).

How does the action of the diterpene, forskolin, fit into these two models? Gilman (51) suggested that the ambiguous results previously observed concerning forskolin did not allow an interpretation of the mechanism of forskloin with his model. These results had been provided by Seamon and Daly (139) and Darfler et al (25). Seamon and Daly (139) had performed studies on the effects of forskolin in cells of the mutant cyc^-S49 murine lymphoma cell line. These cells lack a functional guanine nucleotide regulatory protein (13,73). Using these cyc^-S49 cells, Seamon

and Daly found that forskolin could stimulate adenylate cyclase activity, even in the absence of G_s (138). In contrast, Darfler et al (25) reported that C enzymatic activity was significantly higher with mixtures of C, G_s, and forskolin than with mixtures of forskolin and C alone. An explanation to resolve this dilemma can be supplied by the model of Skorecki et al (144). Forskolin in this model possibly prolongs the activation of C, even after it dissociates from the activated α_{GTP} subunit of G_s. Therefore, though forskolin may not directly stimulate the enzymatic activity of C, it results in a greater production of cAMP (144). Similarly, the Skorecki et al model would attribute the stimulation of C activity observed with the addition of fluoride to an increased dissociation of the $G_s\alpha_{GTP}$-C complex, resulting in a greater amount of activated C (144). The metabolism of the cAMP, which results from the enzymatic action of AdC, is thought to be modulated by a system wholly independent of AdC itself.

1.1.2.3 Metabolism of cAMP -- In sensitive epithelia, the result of administering a stimulating dose of vasopressin is an increase in intracellular cAMP, both in cells of the mammalian collecting duct (5,38,40,54,60,72), and in toad bladder (61,116). However, the overall metabolism of cAMP is under the control of numerous factors in addition to AdC (Fig. 1).

For example, as was mentioned above, several substances, such as forskolin, can affect the enzymatic activity of AdC in the production of cAMP. In the case of forskolin, a stimulation of cAMP production is observed (25,110,138,139,148). In contrast, α -adrenergic agonists have been shown to inhibit the generation of cAMP, probably by stimulating G_i (51). Similarly, it has been reported that PGE_2 inhibits vasopressin-dependent cAMP generation (162). However, all of these studies

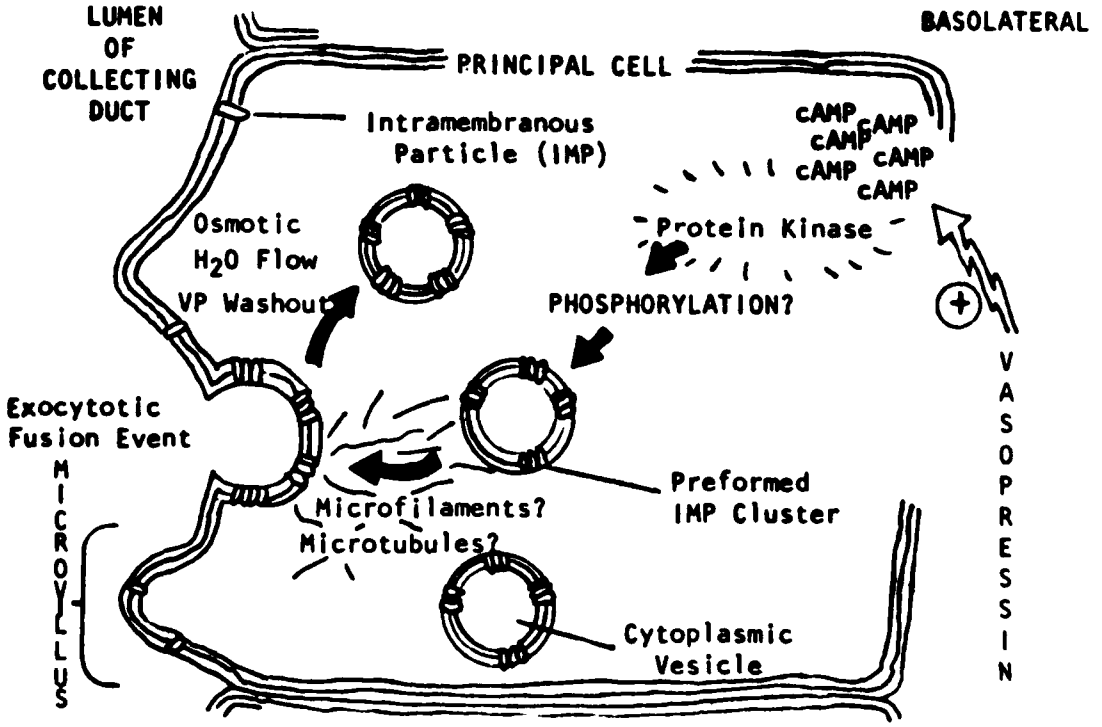
presuppose that there is a sufficient amount of substrate for enzymatic conversion by AdC to cAMP. A deficiency of the substrate, ATP, can result in a failure to accumulate physiologically effective intracellular concentrations of cAMP (37,38).

In addition to factors that affect the generation of cAMP, there are also at least two factors which may decrease cAMP after it has been generated. The cAMP generated may be lost through efflux (41). No studies have been done to date on this phenomenon in the mammalian collecting duct system. However, it is not unreasonable to assume that some intracellular cAMP could be lost through efflux, especially during the periods of high transepithelial water flow (L_p). Secondly, specific isoenzymes (157), collectively termed cyclic-3',5'-AMP phosphodiesterase (PDIE), catalyze the hydrolysis of cAMP to 5'-AMP (4). It is not known at present whether, in vasopressin-sensitive epithelia, PDIE is under direct modulation or feedback control by other cellular enzymes. However, there is evidence that the substrate for PDIE, ATP, is an inhibitor of PDIE activity (31). It is also possible that adrenal steroid hormones inhibit PDIE activity, both in toad bladder (157) and in isolated, perfused, cortical collecting ducts from rabbits (137). Adrenal steroids also reduce the adenylate cyclase response to vasopressin in rat medulla by decreasing vasopressin receptors and by interfering with AdC-receptor coupling (127). In addition, numerous investigators have reported that various natural methyl xanthines and synthetic analogs, such as 1-methyl-3-isobutyl xanthine (MIX), inhibit PDIE. These agents allow intracellular cAMP levels to increase, which results in an increased functional response (37,54,55,61,116). Under normal conditions, however, PDIE activity does not result in conversion of enough cAMP to 5'-AMP to totally eliminate the functional response. Rather PDIE appears to be an integral part of normal cAMP metabolism

within the cell (41).

1.1.2.4 Other Intracellular Mechanisms -- The main thrust of the present investigation concerns morphological changes observed in luminal membranes of cells from collecting ducts of mice under vasopressin stimulation. However, there are numerous intracellular responses to increased cAMP (Fig. 2). For example, several investigators have reported that increased intracellular cAMP caused an increase in protein kinase activity in frog bladder (148), toad bladder (135), and in medullary tissue of mammalian kidneys (32,43,136). While the consequences of increased activity of protein kinase in these systems remain obscure, they may include mediation of the insertion of preformed intramembranous particle clusters from the membranes of vesicles into luminal membranes (Fig. 2).

Figure 2. A model of the cellular events in principal cells that are distal to an increase in cAMP.



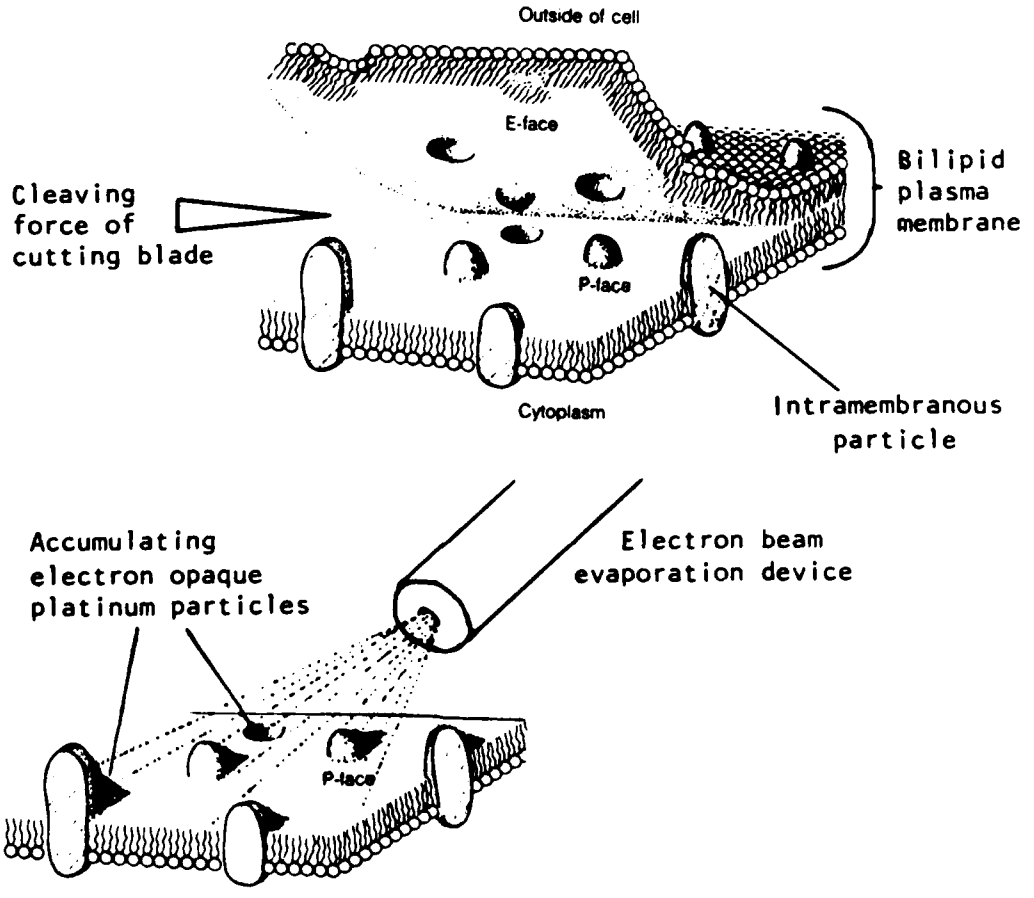
The responses of some of the cytoskeletal elements in cells of vasopressin-sensitive epithelia are also affected by intracellular concentrations of cAMP. In numerous studies on toad bladder, the addition of cytochalasin B, an agent that interferes with the function of microfilaments, has significantly reduced osmotic water flow (26,27,62,78,93,108,120,159). Also in toad bladder, colchicine, an agent that disrupts the function of microtubules, inhibited the hydroosmotic effect of vasopressin (78,108,128,159,160). Dousa and Barnes have reported that colchicine produces the same effect in the mammalian kidney (38). While there is general agreement that intracellular microtubules and microfilaments must function correctly in order for vasopressin-sensitive epithelia to mount an effective hydroosmotic response, the roles that these cellular organelles have in the response remain unclear. Some investigators have suggested that they may control the distribution of IMPs in luminal membranes of these epithelia (78,120).

1.1.2.5 Intramembranous Particle (IMP) Clusters in Anurans -- Early investigators of the hydroosmotic response in epithelial cells of toad urinary bladder had demonstrated, by thin section electron microscopy, that cAMP formation was accompanied by morphological changes in the luminal membranes of these cells (61,116). Chevalier et al (22) and Kachadorian et al (74) pioneered the use of freeze fracture electron microscopy to further examine these changes that occur after tissues are exposed to neurohypophysial hormones. In the freeze fracture technique (see Materials and Methods, and Fig. 3 for details), small samples of tissue are frozen in liquid nitrogen and subjected to a shearing force. The frozen cells fragment at the weakest interfaces of their molecular structure. One such interface is the hydrophobic center between the two parts (leaflets) of the phospholipid bilayer of the plasma membrane

(14,28). The opposing fracture faces of the membrane leaflets may then be coated (shadowed) with platinum particles at an angle of approximately 45° to the tissue surface, followed by carbon particles at 90°. The platinum particles provide image contrast since they do not allow the passage of electrons during electron microscopy. The carbon adheres to the platinum, resulting in a durable "mold" of the fracture face of the tissue. This mold is called a replica.

The replica can then be examined by electron microscopy. During the shadowing process, vertical surfaces will accumulate platinum particles, which will appear as shadows on the screen of the electron microscope, or as darker areas on photographs produced from the microscope. Because the

Figure 3. Diagram illustrating the cellular and molecular basis for the freeze fracture technique.



plasma membrane is a bilayer, either or both surfaces are potentially available for observation and analysis. The leaflet of the membrane bilayer adjacent to the protoplasm is designated the P face, while the leaflet from the cell surface is designated the E face. It is generally accepted that the images of small, globular particles, which sometimes appear on freeze fracture replicas, correspond to integral membrane proteins (97,124,143,163,164,174). There is some evidence, at least in human erythrocytes, that the protein particles may function as pores to allow the transmembrane passage of hydrophilic molecules (126). These particles, which presumably span the membrane, have been termed intramembranous particles (Fig. 3).

Particles of this nature were observed by both Chevalier et al (22) and Kachadorian et al (74), who employed the freeze fracture technique to examine apical membranes of anuran bladders. Such bladders are analogous to mammalian collecting ducts in their response to neurohypophysial hormones, and are often used as model systems for the mammalian distal nephron. For example, sensitive cells of the toad bladder respond to an increase in vasopressin with a rise in intracellular cAMP (117), as well as an augmentation of luminal membrane water permeability (29). Both Chevalier et al and Kachadorian et al showed that increased transepithelial water flow (L_p) across these anuran bladders in response to neurohypophysial hormone was accompanied by morphological changes in the apical surfaces of the bladders. Chevalier et al reported the appearance in frog urinary bladder, of greatly increased patches of what they called "membrane-associated particles" when the cells were stimulated with oxytocin (22). Kachadorian et al described a similar phenomenon, which they called "intramembranous particle aggregation", in toad bladder luminal membranes stimulated by vasopressin (74).

Although the terminology used by both groups of investigators to

describe the particles was different, the descriptions of the particles themselves were not. Neurohypophysial hormone-stimulated cells of both toad bladder and frog bladder had IMPs grouped in linear arrays on P faces, with parallel linear depressions on E faces.

Data from three different studies provided evidence that the clustering response was related to increased water permeability in these cells. Kachadorian et al demonstrated that the clustering response in toad bladder was connected with that action of vasopressin which induced osmotic water flow, and not with urea or sodium transport (75). The previous study by Kachadorian et al (74), which originally described the IMP clustering phenomenon, had also demonstrated that a strong correlation existed between IMP cluster frequency and vasopressin-induced osmotic water flow. In addition, experiments by Brown et al (18) in toad skin, another vasopressin-sensitive tissue, revealed a similar correlation.

1.1.2.6 Intramembranous Particle (IMP) Clusters in Mammals -- Harmanci, Kachadorian, Valtin and DiScala used the freeze fracture technique to assess more directly the water permeability of collecting ducts in mammals. For their first experiment (63) they used the Brattleboro rat, because untreated homozygotes of that strain have no measurable vasopressin (165), and therefore could be predicted to have a very low water permeability and thus, perhaps few, if any, intramembranous particle (IMP) clusters. On the other hand, homozygotes given vasopressin, a treatment known to increase the water permeability of mammalian collecting ducts (54,106,130), might have many IMP clusters. The studies demonstrated that homozygous Brattleboro rats treated with exogenous vasopressin showed a significant increase in IMP clusters within the luminal membranes of medullary collecting duct cells (63).

Urine osmolality was also significantly higher in animals with increased clusters. The data thus showed that, as in anuran membranes, vasopressin caused IMP clustering in mammalian collecting duct apical membranes. However, the evidence to support an obligate relationship between the IMP clustering response, water permeability, and U_{osm} was still indirect.

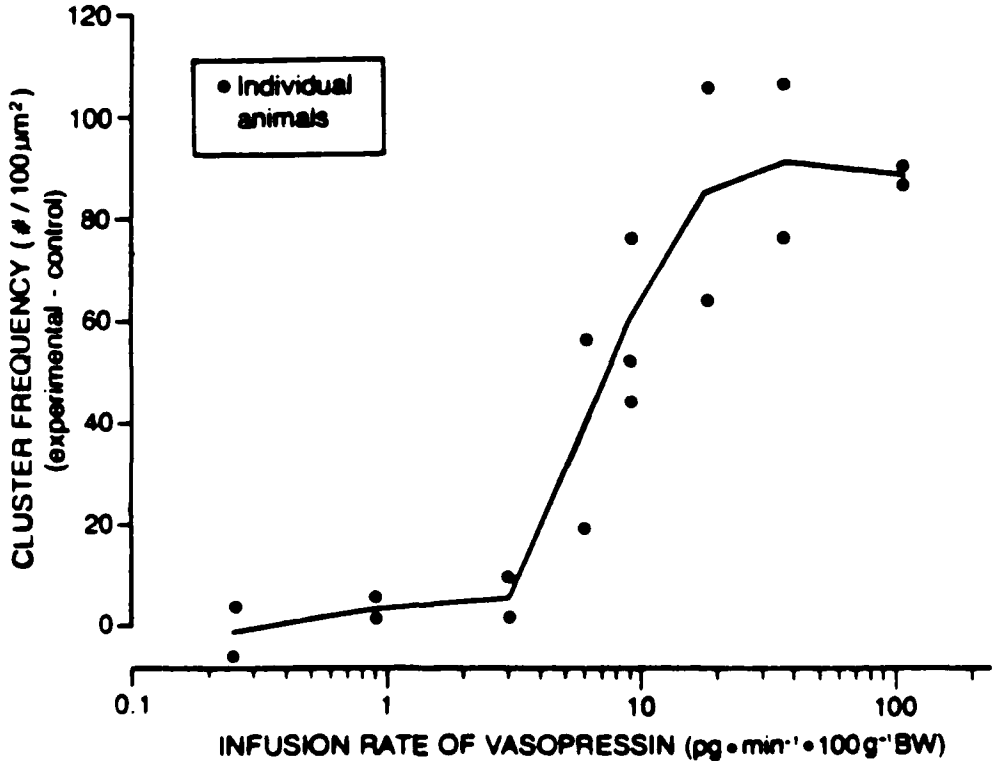
Subsequent investigations with the Brattleboro rat by Harmanci et al (64) and Stern et al (151) demonstrated comparable results, with excellent correlations between cluster frequency, dose of vasopressin, and urinary osmolality. Furthermore, experiments by Lacy with the Hannover-Wistar rat in various states of water diuresis and antidiuresis, verified the relationship, which had been previously shown in the Brattleboro rat, between cluster frequency and U_{osm} (92). These results, combined with those from previous studies, meant that neurohypophysial hormone-sensitive epithelia from two different strains of rat and two different anurans had responded to vasopressin in a similar manner: increased osmotic water flow or increased U_{osm}, presumably reflecting increased water permeability, accompanied by parallel increases in the frequency of IMP clusters.

Similar results have been reported recently for human tissue. In unkeratinized human fetal skin incubated in vitro (gestational age 9-14 weeks), Riddle has documented IMP clustering in response to an exogenous, second messenger, dibutyryl cyclic AMP (129). However, Riddle made no attempt to assess whether this morphological effect was accompanied by increased osmotic transepithelial water flow (L_p), although neurohypophysial hormones have been shown to increase L_p in fetal skin of two other mammals. Holt and Perks (68) showed that the skin of the fetal guinea pig increased unidirectional osmotic water flow in vitro in response to vasopressin. France (49) documented a similar response in the skin of fetal sheep.

Critical analysis of the two previous experiments with Brattleboro rats (63,151) suggested that the responses of augmented osmotic water flow and parallel increased frequency of IMP clusters might be inseparable. In the Brattleboro rat any acute increase in osmolality of the urine following exposure to vasopressin must be regarded as the result of an increase in collecting duct water permeability (168). Furthermore, the Brattleboro rat responds to vasopressin by increasing IMP clusters and U_{osm} in a dose-dependent fashion (64)(see Fig 4). The data imply strongly that water permeability and IMP clusters are coincident.

1.1.2.7 Source of IMP Clusters in Anurans -- In 1972 Masur et al (99) reported that stimulation with neurohypophysial hormone caused exocytosis

Figure 4. A dose-response curve relating the amount of vasopressin given to Brattleboro rats and the intensity of IMP clusters in medullary collecting ducts of the same animals. IMP cluster frequency is the mean of experimental kidney minus the mean of control kidneys, namely 3 ± 1. The line is drawn through mean of cluster frequencies observed at each dose of vasopressin. (Reproduced from Harmanci et al [64]).



of cytoplasmic granules in granular cells of the toad urinary bladder. This finding had closely followed the discovery in the previous year by the same group, that these hormones, as well as exogenous cAMP, caused pinocytosis and increased water permeability in the same type of cells (98). The data from these studies led these investigators to postulate that membrane from membrane-bound granules was added to the effector (apical) surface of the cells as a consequence of the exocytosis of the granules -- a maneuver that materially altered the transport properties of the apical membrane. Evidence supporting the part of their hypothesis which pertained to membrane addition was provided by several other researchers during additional experiments on the IMP clustering response in the toad bladder. Theories on the source of this added membrane, however, remained controversial.

Two laboratories independently reported that IMP clusters were present not only in apical membranes but also in cytoplasmic "vacuoles" or vesicles (69,171). Humbert et al (69) reported IMP clusters in cytoplasmic vesicles, which very closely resembled the IMP clusters found in the luminal membrane. The clusters from the vesicles had the same characteristic, parallel rows of particles in the P face, and complementary parallel grooves in the E face as were observed in clusters from the luminal membrane. These workers had observed this phenomenon both in bladders from control animals, which did not have IMP clusters in their luminal membranes and in vasopressin-treated animals, which did have IMP clusters in their luminal membranes (69). Wade also reported the presence of IMP clusters in the membranes of cytoplasmic "vacuoles" or vesicles in bladders from hydrated toads, which did not have IMP clusters in their luminal membranes (171). According to Wade it was easy to distinguish these "vacuoles" from the granules which Masur et al (99) had identified earlier. He found that the "vacuoles" had IMP clusters

embedded in their membranes, while the granules did not. The fact that these IMP clusters appeared to be identical to the IMP clusters observed in the luminal membranes led both Humbert et al (69) and Wade (171) to speculate that the "vacuoles" or vesicles could be the source of the intramembranous particles found in the luminal membranes.

In 1980, Wade reviewed experiments conducted on the sources of IMPs (172). He hypothesized that there could be three possible sources for the aggregates, or clusters, of particles: (a) lateral movements or coalescence of particles already dispersed in the membrane; (b) insertion of "new" individual particles into the membrane; or (c) insertion or fusion of cytoplasmic, vacuolar membranes which contained preformed aggregates. If (a) or (b) were the case, then, he argued, it would be probable that IMP aggregates should vary in size according to the time elapsed from initial vasopressin stimulation. Experiments by Wade's colleagues Kachadorian et al (77) showed, however, that aggregate size did not increase with the duration of vasopressin stimulation, nor did the incidence of smaller aggregates. Furthermore, earlier experiments by Kachadorian et al (76) showed that the frequency of intramembranous particles not located in clusters, did not increase with vasopressin stimulation, suggesting that the insertion of "new" IMP clusters, rather than individual particles, was responsible for the increase in the permeability of the membrane. Finally, Wade himself found that the granular cells from vasopressin-stimulated bladders contained fewer of the aggregate-containing vacuoles in the cytoplasm than cells from unstimulated bladders, suggesting that vasopressin had caused exocytosis and subsequent disappearance of these vacuoles from the cytoplasm (172). Therefore, Wade suggested that hypothesis (c), the insertion of cytoplasmic, vacuolar membranes, containing preformed IMP aggregates, was the logical choice.

Now, armed with evidence that IMP clusters could be found preformed in cytoplasmic vacuoles [two studies previously mentioned (69,171) plus a new, confirmatory study (42)], Wade proposed the "Membrane Shuttle Hypothesis" (172), a variation of the hypothesis proposed by Masur et al (99). Wade's hypothesis stated that vasopressin caused the translocation and fusion of vacuoles with the luminal membrane, inserting aggregates of IMPs, which increased water permeability. When vasopressin concentration decreased, patches of luminal membrane would be retrieved by endocytosis with IMPs intact. To Wade, the process of retrieval and insertion of preformed aggregates of IMPs within vacuoles resembled a similar process used by the NASA Space Shuttle to insert and retrieve objects from space (172).

At about the time Wade's review (172) appeared, Chevalier et al (23) provided an additional piece of evidence that Wade could have used to support his own hypothesis. As three studies (42,69,171) before theirs had reported, Chevalier et al (23) also found cytoplasmic vesicles in toad bladder that contained IMP clusters with the same morphology as those from the apical membrane. Water permeability in these bladders closely paralleled the appearance of IMP aggregates or clusters in the apical membrane (23). In addition, in carefully controlled time studies, Chevalier et al were able to document the appearance of IMP aggregates within 1 minute of vasopressin stimulation (23). In light of the previous findings of Kachadorian et al (77), to the Chevalier group this seemed to be hardly enough time for any mechanism except insertion of membrane-containing preformed IMP clusters (23). Actually, this time limit probably excludes only de novo synthesis of the particles. For example, low density lipoprotein (LDL) receptors in apical membranes of cultured human fibroblasts exist in two configurations: clustered in coated pits and diffusely distributed over the membrane surface (1,2).

Experiments by Anderson et al suggested that these LDL receptors can move in the membrane from the diffuse configuration to the clustered configuration within 6 minutes (1,2). Since the time course of the LDL receptor clustering is of the same order of magnitude as the time stipulated in the experiments of Chevalier et al (23), rapid IMP clustering probably had not been ruled out by the rapidity of the response.

By 1980, both the Wade (Kachadorian) and Masur groups had further morphological evidence to support the concept of membrane "shuttling" or cycling, although their theories invoked the operation of different mechanisms. Evidence supporting the Wade hypothesis of insertion of membrane into vasopressin-stimulated toad bladder was provided by Muller, Kachadorian, and DiScala (108), who were able to confirm the existence of the cytoplasmic, vacuolar structures containing the characteristic arrays of IMP's, which had been described by Humbert et al (69) and Wade (171). Further, in favorable freeze-fracture replicas, Muller, Kachadorian, and DiScala were able to show fusion events of these vacuolar structures, which they had dubbed "aggrephores", with the luminal membrane, as well as a linear correlation between fusion events and the number of IMP aggregates observed (108). These new data, when coupled with previous findings (23,69,171), provided strong evidence for the aggrephore-shuttling theory. Bourguet et al, in their review of 1981, strongly supported the hypothesis that vasopressin stimulation in toad bladder caused the insertion of membrane with preformed IMP clusters into luminal membranes (11). They have subsequently reiterated this support in an abstract (12). Hays also supported this view in his review of 1983 (67).

Gronowicz, Masur, and Holtzman (57) provided morphological evidence in support of the Masur hypothesis when they reported retrieval as well as insertion of membrane in response to vasopressin stimulation. Their

data showed that toad bladder granular cells under the influence of vasopressin had increased membrane endocytosis, as well as increased granule exocytosis (57). They also found that the time course for the disappearance of granules from the cytoplasm correlated well with the time course for the increase in hydroosmosis. Further, their calculations for the amount of membrane retrieved by endocytosis was similar to (at least 3/4 of) the amount of membrane exocytosed over a one-hour period. In addition, colchicine, an agent that disrupts the function of microtubules, inhibited the hydroosmotic response to vasopressin while simultaneously inhibiting both the exocytosis of granules and the subsequent endocytosis of luminal membrane. Their conclusion was that the insertion of membrane was due to the exocytosis of granules (rather than IMP-containing vacuoles, or aggrephores). Thus, exocytosis and subsequent endocytosis of membrane resulted in what they termed "membrane circulation", which they found to be related to changes in water permeability of these cells (57).

In contrast, several groups have been unable to duplicate the results of the Masur group which showed exocytosis of granules rather than aggrephores in the granular cells in response to vasopressin, including Muller et al (108), Wade (172), and Reaven et al (128). Masur responded to their contrary findings during the discussion session of one of Wade's presentations (173). She argued that the failure of these investigators to demonstrate similar results might have been attributable to the different degrees of stretch given to bladders during preparation of the tissues. In support of this argument, Masur cited the results of Brown et al (16) concerning the effects of stretch on exocytosis and IMP clusters. Brown et al (16,17) had found that granular cells could exocytose up to 50% of their granules during a 5-minute period of stretch. Interestingly, they also found increased IMP clusters in these

bladders, which were stretched but not treated with exogenous neurohypophysial hormone (16). It should be noted, however, that Kachadorian and Levine could not duplicate these results (80).

Subsequently, the morphological evidence for the portions of each hypothesis that concerned membrane insertion was supplemented by other types of data. These data came from two different laboratories in experiments designed to measure electrical properties of toad bladder. Stetson et al (154), using voltage relaxation techniques, were able to measure a 30% increase in transepithelial capacitance with vasopressin stimulation. They had used various experimental manipulations to eliminate all known sources for increased capacitance except for increased surface area. Their results therefore suggested that the increase in capacitance was most likely due to the addition of membrane to the apical membrane. They then attempted to confirm the capacitance results with a parallel analysis of membrane morphometry. However, even though they measured a 29% increase in mean apical membrane surface area, the change was not statistically significant due to the large variability of the response between bladders. Palmer and Lorenzen (118) later demonstrated a 28% increase of capacitance in toad bladder stimulated by the same concentration of vasopressin that had been used by Stetson et al (20 mU/ml)(154). These data also implied that a significant increase in membrane surface area had occurred. In addition, Palmer and Lorenzen showed that the time course for increase of vasopressin-stimulated water flow was similar to the time course for increase in capacitance. Finally, they demonstrated that both cytochalasin B, an agent that interferes with the function of microfilaments, and colchicine, one that intereferes with microtubules, inhibited not only the increase in capacitance in vasopressin-stimulated bladder, but also the increase in water flow (118).

However, final arguments for both the Masur hypothesis of granule exocytosis and the Wade hypothesis of aggrephore exocytosis, were not yet all in. In fact, members of both groups supplied data, which, at least in part, substantiated the hypothesis of the other. In 1984, Muller and Kachadorian (109) confirmed their earlier studies in toad bladder, which suggested that vasopressin stimulation caused fusion of tubular aggregate-containing structures, or "aggrephores", with the luminal membrane. They also confirmed that endocytosis followed closely the washout of vasopressin, because horseradish peroxidase (HRP), which had been applied to the luminal surface, was found inside numerous aggrephores after washout. Additionally, bladder cells subjected to vasopressin washout had an increased population of multivesicular bodies, most of which contained HRP, suggesting that membrane and/or aggrephores had been retrieved, and were about to be "processed" (109). With the exception of the exocytosis of granules, these findings were in obvious agreement with those of Gronowicz et al (57) mentioned previously.

Furthermore, the measurements of Muller and Kachadorian showed that vasopressin stimulation did not increase planar surface area of these stimulated cells, so that the increased effective luminal membrane area had to result from the addition of vertical surface area from the walls of the fused aggrephores (109). Levine et al (94) have provided some support, at least in theory, that this is most likely the case.

One of the main problems associated with explaining the increase in water permeability found in membranes in response to neurohypophysial hormones, is that the membrane permeability to small ions does not increase in parallel. The ratio $[P_f/P_d(w)]$ is an expression of the relationship between P_f, the osmotic water permeability coefficient and $P_d(w)$, the diffusional water permeability coefficient. The value of this ratio provides information on the nature of the pore radius of the water

pathway. A theoretical study by Levine et al (94) using this ratio, proposed three models which attempted to reconcile the previously mentioned differences between the impermeability of the water pathway to small ions and the obvious increase in water permeability proper. They presented compelling arguments, which suggested that, of the three models, the data calculated for the fused aggrephore model best fit the $[P_f/P_d(w)]$ ratios that have been observed experimentally.

Masur et al (100) published a paper which was somewhat conciliatory to Wade's theory of aggrephore shuttling. They reaffirmed that vasopressin stimulation in toad bladder caused exocytosis of granules from granular cells, but conceded that the membranes of the cytoplasmic granules did not contain the preformed IMP clusters. They also confirmed that tubular aggregate-containing structures (Muller and Kachadorian's "aggrephores") fused with the luminal membrane, and probably contributed to increased membrane water permeability (100). Furthermore, Masur et al determined that these fused structures detached even in the presence of vasopressin, if there was no osmotic gradient, and, similar to what Muller and Kachadorian had simultaneously reported (109), were endocytosed during vasopressin washout, or if an osmotic gradient were imposed (100). These results suggested to Masur et al that either the absence of a gradient inhibited endocytosis, or that the presence of a gradient enhanced endocytosis. The results also provided a credible explanation (100) for the equivocal results of the three earlier studies of Ellis et al (47), Palmer and Lorenzen (118), and Stetson et al (154).

Two of these previous studies, that of Stetson et al (154) and that of Palmer and Lorenzen (118), had found increased capacitance in vasopressin-stimulated bladders with no osmotic gradient (see above). The new report of Masur et al (100) suggested that this was because "normal" endocytosis, which would have moderated the increase started by

vasopressin-stimulation, was not taking place. Similarly, the data of Masur et al (100) helped explain the data of Ellis et al (47). This study had shown an increase in IMP clusters in vasopressin-stimulated bladders not subjected to an osmotic gradient when compared to the IMP clusters observed in bladders subjected to a gradient (47). The IMP clusters in the bladders not subjected to a gradient were not being removed or reduced by endocytosis (100). From a consideration of these studies (47,118,154), and the interpretation of their own data, Masur et al then proposed that in the presence of an osmotic gradient, the hydroosmotic effect may alter water-permeable portions of the membrane so that these portions undergo endocytosis. Subsequently endocytosis may "obscure and temporarily inactivate channels" to help restore the more impermeable nature of the apical membrane (100). In their latest report, Masur et al reiterated that the exocytosis of granules as well as tubules (Kachadorian's aggrephores), elicited by antidiuretic hormone causes an increase in apical membrane surface (101). They also have begun to analyze isolated granules and their membranes, and report that the membranes of granules are not of the same composition as what they have termed "average", unstimulated, luminal membrane. Further, they speculated that if stimulated luminal membrane had to have different properties from unstimulated membrane in order to mediate an increase in water permeability, the different composition of granule membranes could fulfill this requirement (101).

1.1.2.8 Source of IMP Clusters in Mammals -- Investigations concerning the source of IMPs in mammals have only recently begun. Data from experiments by Brown and Orci (19) have suggested a direction that such investigations might take.

In 1976 Pearse demonstrated that membranes with coated vesicles, as

evidenced by the presence of bristle coats composed of clathrin, had a low cholesterol to phospholipid ratio (122). In 1979 Elias et al (46) showed that the antibiotic filipin could be used as a cytochemical probe for membrane cholesterol. Montesano et al (104) subsequently used filipin to probe the membranes of several types of cultured cells for cholesterol. They found that filipin-sterol complexes were missing from depressions that were associated with bristle coats, which strongly suggested that filipin could be used to identify coated pits.

Orci et al also used filipin to probe luminal membranes of granular cells from toad bladder (114). They found that filipin-sterol complexes were absent from areas of the membranes that contained IMP clusters, which had been induced by vasopressin stimulation (114). In this study they made no attempt to identify whether there were bristle coats associated with the IMP aggregates. While Orci et al suggested that the IMP aggregates could be the sites of specialized permeability, they conceded that the "tightness" of the particles within the aggregates might have sterically inhibited filipin-sterol complexes from forming (114). The same IMP aggregates in toad bladder, however, had been shown to be closely correlated with osmotic water flow (74,75). It was possible that the same relationship between IMP aggregates and coated pits existed in other epithelia that were sensitive to vasopressin.

Brown and Orci used freeze fracture and thin-section electron microscopy on luminal membranes from principal cells of collecting ducts in Wistar (normal) and Brattleboro (hypothalamic diabetes insipidus) rats to investigate that possibility. The Wistar rats were deprived of water for 24 hours, a treatment known to induce the formation of IMP clusters (63,64). Brattleboro homozygous rats were examined, either untreated as controls, or treated with daily injections of vasopressin. Sections of inner medulla from both the Wistar and Brattleboro rats were incubated

overnight in the presence or absence of filipin. All of the tissues were then processed appropriately for freeze fracture or thin-section electron microscopy.

Sections from both the Wistar rats and the vasopressin-treated Brattleboro rats had numerous IMP clusters. Transmission electron microscopy of freeze fracture replicas demonstrated that the areas of the membranes which had aggregates of IMP clusters did not show filipin binding. Further, thin-section electron microscopy demonstrated that regions of the plasma membrane that had a cytoplasmic bristle coat also had no filipin binding (19). The aggregates of IMPs that were observed in these rat collecting duct cells were much less dense than those that had been observed in the luminal membranes of granular cells from toad bladder. Steric hinderance could not have caused a problem in forming filipin-sterol complexes in these tissues. Since two things that are equal to the same thing are equal to each other, there was little doubt for Brown and Orci that the IMP aggregates or clusters and coated pits were equivalent (19). Although they did not speculate on the relationship of IMP clusters and coated pits in mediating water permeability in the collecting ducts of mammals, circumstantial evidence for such a relationship exists.

The morphological features observed by Brown and Orci in both freeze fracture and thin section were consistent with those of endocytotic coated pits, which had been characterized previously in several cellular systems (53,104,113). Coated pits, in most other cells, are involved in receptor-mediated endocytosis (53). However, coated pits have also been implicated in processes which transport vesicles to the cell membrane from the cytoplasm (3,50,52,96,132,133). Since IMP clusters have been found to be coincident with coated pits in principal cells from both Wistar and Brattleboro rats, it is not unreasonable to

hypothesize that the coated pit mechanism may be a part of a membrane shuttle mechanism for IMP clusters in mammals.

1.2 Defects of Urinary Concentration

1.2.1 Possible Mechanisms -- The first section of the Introduction described the normal processes within the mammalian nephron that maintain water balance and enable urine to be concentrated to an osmolality higher than plasma. In mammals, there are clinical syndromes in which the urine cannot be concentrated sufficiently, so that a constant threat to normal water balance ensues. One such syndrome called diabetes insipidus is characterized by the production of large volumes (diabetes) of dilute or "tasteless" (insipidus) urine. Keeping in mind the normal operation of the nephron previously described, diabetes insipidus could conceivably be caused by the presence of, among others, one or more of the following defects: (A) failure to produce a sufficient corticopapillary osmotic gradient; (B) failure to release vasopressin when a water deficit is present in the animal; or (C) failure of vasopressin to increase water permeability of cells lining the collecting duct system.

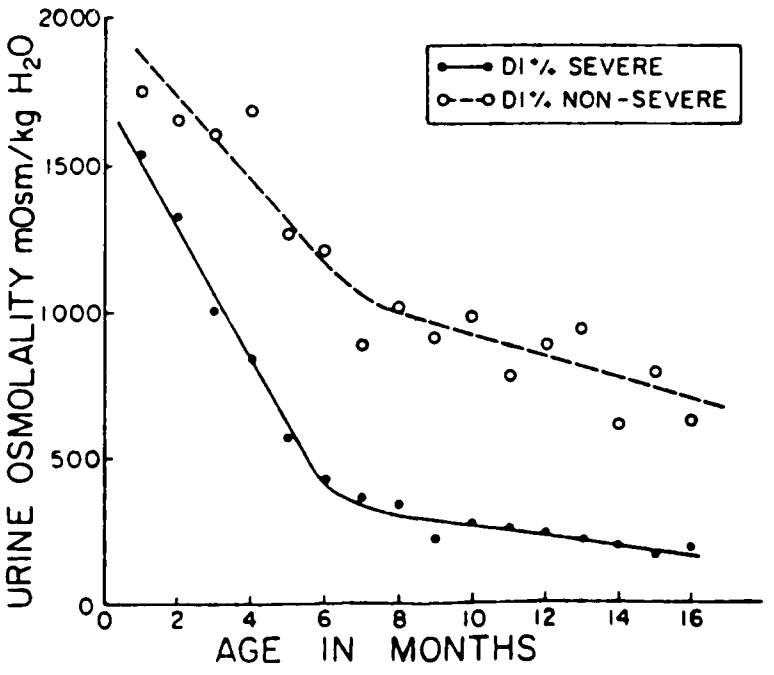
1.2.2 Animal Models

1.2.2.1 Hypothalamic Diabetes Insipidus -- Extensive investigations were carried out beginning in the 1960's with a strain of rat, which produced copious amounts of urine that was hypotonic to plasma, and drank large volumes of water. Valtin and his coworkers (168) had shown that these rats were unable to elaborate vasopressin in response to appropriate stimuli. In addition, rats homozygous for this type of diabetes insipidus had very little vasopressin neurosecretory material (146), and no detectable vasopressin present in their pituitary glands (165). That is, these animals, now called Brattleboro homozygous rats,

exhibited defect (B) above, a failure to respond with a release of vasopressin to appropriate stimuli. This defect is termed hereditary hypothalamic or central diabetes insipidus, and can be corrected by the administration of exogenous vasopressin (65).

1.2.2.2 Nephrogenic Diabetes Insipidus (NDI) -- In 1964 Falconer et al (48) identified a strain of mice as "cage-wetting" mice because they produced large amounts of urine and had a high water intake. The defect in urinary concentration observed in these mice, unlike that in Brattleboro homozygous rats, was not hypothalamic or central. Studies with the "cage-wetting" mice by Naik and Valtin (111), and others (112,155,156) demonstrated that the hereditary concentrating defect of these mice could

Figure 5. Decline in urine osmolality of DI +/+ Severe and DI+/+ Nonsevere mice with age. Each circle or dot represents mean urine osmolality at any given age, of an average of ten mice. [Taken from Valtin (167)]



not be corrected by the administration of vasopressin, and was probably, therefore, nephrogenic in origin. Consequently, the focus of investigation for the source of the defect(s) in these mice was shifted from (B) the failure to release vasopressin, to (A) the corticopapillary gradient, and to (C) the state of water permeability of the collecting duct system of these mice.

By 1971, these "cage-wetting" mice, which Falconer (48) had named DI +/+ for Diabetes Insipidus homozygous, had passed through many generations in Valtin's Dartmouth laboratory. Investigators there had noticed two populations among these mice, separable according to their varying abilities to concentrate urine. In addition, the process of urinary concentration seemed to be partially related to age (167). Animals which after birth could concentrate their urine to normal levels of tonicity, lost that ability by an average age of six months (Fig. 5).

By six months, one genetically definable population, called DI +/+ Severe, excreted urine hypotonic to plasma, with a mean concentration of approximately 150 milliosmoles per kilogram of water (mOsm/kg H_2O). The other genetically definable population, called DI +/+ Nonsevere, had a mean urine osmolality of approximately 1500 mOsm/kg H_2O (85) -- about 1300 mOsm/kg H_2O less than the urine of most normal strains of mice. Was the inability to concentrate urine due to a failure of the tubular fluid to equilibrate with the interstitium [Defect (C) above]? A comparison of the osmolality of the papilla with the osmolality of the urine could help provide the answer to this question.

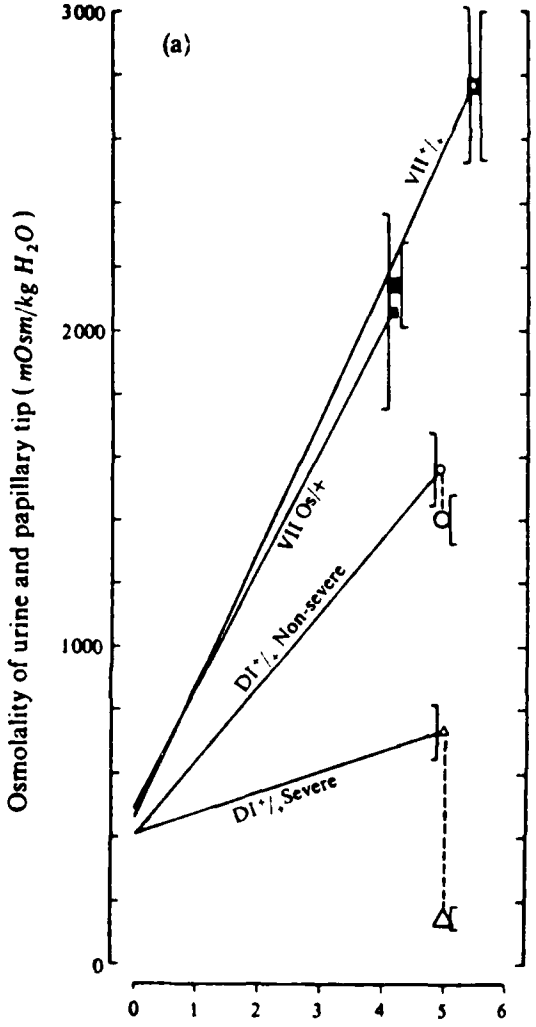
Valtin (166) had previously used a simple qualitative method with the Brattleboro rat to compare the osmolality of the renal medulla and papilla to the osmolality of urine. A comparison of the summation of osmoles due to Na^+, K^+, NH_4^+, and urea concentrations at the tip of the inner medulla versus that of the urine, had readily demonstrated a lack

of osmotic equilibration between the two, presumably due to the absence of physiological levels of endogenous vasopressin. A technique with greater precision was required in order to refine the location of the osmotic disequilibrium.

Valtin with Kettyle (85) modified his earlier osmole-measuring technique (166) by adding dimensional analysis in order to estimate the medullary solute concentration at the very tip of the inner medulla. The existence of any significant difference in the measured osmolality of the urine and the extrapolated osmolality of the papilla would imply that a defect existed in osmotic equilibration between collecting duct fluid and papillary tissue. The finding that U_{osm} and papillary osmolality were not different in normal VII +/+ mice (Fig. 6) -- in other words, that osmotic equilibration had occurred -- validated the experimental technique. The results (Fig. 6) also showed that osmotic equilibration had not occurred in the DI +/+ Severe mice, strongly suggesting defective water permeability in the papillae.

Urine osmolality, U_{osm}, for the control mice (strain VII +/+), was 2764 $\pm$232 mOsm/kg H_2O, while papillary osmolality was 2771 $\pm$246 mOsm/kg H_2O, values that reflect osmotic equilibration. The urine and papillary osmolalities of the VII Os/+ mice demonstrated a similar relationship: 2141 $\pm$138 mOsm/kg H_2O and 2065 $\pm$312 mOsm/kg H_2O, respectively. In contrast, U_{osm} of the DI +/+ Severe mice was 149 $\pm$19 mOsm/kg H_2O, while papillary osmolality was 740 $\pm$85 mOsm/kg H_2O. This apparent inability to achieve osmotic equilibration between tubular fluid and interstitium was, in all likelihood, due to deficient water permeability of the collecting duct system. The DI +/+ Nonsevere mice had a U_{osm} of 1406 $\pm$77 mOsm/kg H_2O and a papillary osmolality of 1561 $\pm$114 mOsm/kg H_2O, a difference which, while noteworthy, was not statistically significant (see Fig. 6). Of interest was the fact that both DI +/+ Severe, and DI +/+ Nonsevere

Figure 6. Urine osmolalities and estimated osmolalities at the papillary tip, plotted against the length of the countercurrent system in each group of mice. Small symbols are papillary tip values; large symbols are urinary values. Brackets represent mean ±SEM. Only the difference between DI +/+ Severe urinary and papillary osmolalities is statistically significant (P<0.001). [From Kettyle and Valtin (85)]



mice had corticopapillary gradients of much lower osmolality than the VII +/+ mice (85). No attempt was made to ascertain whether the lower gradient was due to "washout" of solute caused by diuresis, or to a failure of the loops of Henle to deposit sufficient NaCl into the interstitium.

The results on osmotic equilibration had been arrived at somewhat

indirectly, since they were based upon several assumptions: 1) that the corticopapillary gradients were linear; 2) that papillary water content was 80% and increased to 90% in diuresis; and 3) that the mouse papilla approximated a pyramidal shape (85). Nevertheless, the data from this study suggested strongly that DI +/+ Severe mice had deficient vasopressin-induced water permeability in their late distal tubules and collecting ducts (85).

Recall that the urinary concentrating defect observed in the DI +/+ Severe mice could not be corrected by the administration of exogenous vasopressin. Normally, the binding of vasopressin to its receptor leads to the formation of intracellular cAMP (33,117). Cyclic AMP, in turn, mediates the increase in water permeability of distal tubules and collecting ducts (38,54,56,60). Therefore, the disequilibrium might have resulted from a defect at a particular point, or points, in this biochemical cascade. Elements of this pathway in mice with hereditary diabetes insipidus have previously been examined by Dousa and colleagues (36,70,91).

Early studies by Dousa and Valtin of medullary tissue from DI +/+ Severe mice suggested that the lack of response to vasopressin in these animals was due to abnormal metabolism of cAMP. The activity of vasopressin-stimulated adenylate cyclase was lower in DI +/+ Severe mice than in controls (36). However, the same study, which used enzymatic assays of washed membrane fractions from the entire renal medulla, reported that the activity of cAMP phosphodiesterase (PDIE) in the DI +/+ Severe mice was not different from that of controls (36). A later study, in which individual, microdissected nephron segments from the renal medulla were analyzed, revealed that the medullary collecting tubules of DI +/+ Severe mice could not increase cAMP in response to vasopressin stimulation (70), and that this failure was apparently due to an increased activity of cAMP

phosphodiesterase. The fact that the addition of a specific cAMP phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine (MIX), could significantly increase cAMP levels was consistent with this conclusion (70). Adenylate cyclase activity in the medullary collecting tubules was only slightly lower for the DI +/+ Severe mice than for controls. It is probable that the earlier study of Dousa and Valtin (36) failed to identify the elevated PDIE activity because of the heterogeneous population of nephron segments which made up the membrane fraction for their assays. A recent study by Dousa and colleagues confirmed the existence of elevated PDIE and a slightly decreased adenylate cyclase activity in medullary collecting ducts of DI +/+ Severe mice (91).

In the presence of 10^{-6}M vasopressin, the addition of 5 x 10^{-5}M MIX plus 10^{-4}M forskolin overcame the effects of elevated endogenous PDIE and diminished adenylate cyclase activity, respectively, and the addition restored cAMP levels of DI +/+ Severe mice to those of normal mice (91). However, it is not known whether insufficient cAMP accumulation in medullary collecting tubules of DI +/+ Severe mice is the sole cause of their urine concentrating defect.

1.2.2.3 The Oligosyndactyly (Os) Gene -- The existence of the oligosyndactyly (Os) gene in mice was reported by Falconer et al (48) in 1964. Mice with the Os gene manifest the skeletal defect of few and fused toes (58,59,81,85), as well as a vasopressin-resistant concentrating defect. This urinary concentrating defect apparently is a pleiotropic effect of the Os gene (i.e., concerned with the same gene but usually unrelated functionally) (48). When the Os gene is introduced into normal mouse strains, such as VII +/+ or CBA +/+, there ensues a defect in urinary concentration similar in severity to that observed in DI +/+ Nonsevere mice. For example, urine osmolality in animals with the Os gene (Os/+)

averages 1800 mOsm/kg H_2O, compared to approximately 2800 mOsm/kg H_2O for VII +/+ Normal mice (85,111). The fact that this deficiency is not corrected by exogenous vasopressin (111) means that animals with the Os gene have a nephrogenic defect.

Naik and Valtin have demonstrated that Os/+ mice have kidneys which are 40 to 50% smaller than VII +/+ Normal mice (111). These smaller kidneys were subsequently shown to have nephrons which were approximately 20% shorter (85). Additionally, kidneys from the Os/+ mice had significantly fewer nephrons and tended to retain nitrogen, a manifestation of chronic renal failure (111,155). Because the Os/+ mice normally remain in water and solute balance in spite of chronic renal failure, their kidneys probably have an osmotic diuresis per nephron (167). However, unlike the DI +/+ Severe mice, VII Os/+ mice have no disequilibrium between papillary and urinary osmolalities (85)(Fig. 6, p. 32), which implies that they do not have a defect in water permeability of their collecting duct system.

1.3 Questions Investigated in the Present Study

At this point, a summary of the data from anuran studies, distilled into a working hypothesis, would be helpful. In cells which respond to stimulation by neurohypophysial hormone by increasing luminal water permeability, intramembranous particle clusters are inserted into the apical membrane by exocytosis of cytoplasmic, IMP-containing, tubular structures called aggrephores (probably not by the exocytosis of granules). The IMP clusters are probably retrieved by endocytosis in response to removal of the hormone and/or by action of the transcellular osmotic gradient. Water flow in these cells, in response to the same hormone and an osmotic gradient, increases and decreases, by and large, over the same time course, respectively, as exocytosis and endocytosis. It is therefore

highly likely that a membrane "shuttle" mechanism exists, at least in anurans, such that ultimate apical membrane water permeability depends upon the insertion or retrieval of patches of membrane more permeable to water.

Until the present study, there were no data concerning the presence or absence of IMP clusters in mice. However, the IMP clustering response had been demonstrated in two other mammals: in the rat collecting duct system (19,63,64,92,151), and in the skin of the human fetus (129). Therefore, it would not be unreasonable to assume that the cells of the mouse collecting duct system respond to vasopressin-stimulation with the IMP clustering response.

Further, if mice do have the IMP clustering response it would be likely, in accordance with the principle of universality of biological phenomena, that these mice also have a shuttle mechanism. However, there is currently no evidence to support or deny the existence of such a shuttle mechanism in vasopressin-responsive cells other than those of anurans.

Finally, if mice do have the IMP clustering response, the density of the IMPs within each cluster might be similar to the particle density found in anuran cells. At the present time there is no consensus among investigators as to whether the response, even in anurans, is one of an increase in IMPs per cluster, one of an increase in the number of clusters per cell, or one of a recruitment of additional responding cells.

1.3.1 Intramembranous Particle (IMP) Clusters in NDI Mice -- Recall that mice with severe nephrogenic diabetes insipidus had previously been shown to have defects of urinary concentration. Jackson et al (70) reported that medullary collecting tubules from DI +/+ Severe mice,

incubated in vitro, had elevated cAMP phosphodiesterase activity. Additionally, vasopressin-sensitive adenylate cyclase activity in medullary collecting tubules from DI +/+ Severe mice was lower than that observed in normal mice (70). Both of these defects could result in a less than normal accumulation of intracellular cAMP in response to stimulation with vasopressin. Although steps distal to the generation of cAMP remain in question, it is generally accepted that the increase in water permeability observed in responsive tissues is mediated by an increase in intracellular cAMP. This is true for both mammalian cells (5,38,40,54, 60,72) and anuran cells (61,116). Data from anuran studies have demonstrated that the IMP clustering response is closely correlated with water permeability in granular cells (18,74,75) and that the change in water permeability is mediated by an increase in intracellular cAMP (61,116). In addition, Kettyle and Valtin (85) have shown that DI +/+ Severe mice, and possibly DI +/+ Nonsevere mice, have a disequilibrium between papillary osmolality and urinary osmolality (Fig. 6, p. 32), suggesting that a defect in water permeability existed in the collecting duct system of these animals.

These data, when combined with the fact that biologically efficient mechanisms tend to be conserved during evolution, lead to the prediction that the IMP clustering response would be deficient in NDI mice. Hence, the first question of the present investigation:

I. What is the status of IMP clusters in the collecting duct system of NDI mice?

1.3.2 Characteristics of IMP Clusters in NDI Mice -- There have been no studies prior to the present one on the cause of the disequilibrium between urinary osmolality and papillary osmolality in the DI +/+ Severe mice (Fig. 6) (85). Studies on the time course in toad bladder had

revealed that granular cells respond to continuous vasopressin stimulation with an increase in frequency of IMP clusters (23,77). Consistent with this result was the finding of Kachadorian et al (77) that the size of IMP clusters did not increase with duration of vasopressin stimulation. If our assumption is correct, namely, that the intensity of the IMP clustering response in principal cells reflects water permeability of the mouse collecting duct system, one would expect to see a high frequency of IMP clusters in cells from mice with presumed high water permeability. The dose-response data from the previous rat study (64) also suggested that the response would be one of increased number of clusters per cell. However, early experiments in the present study revealed that CBA Os/+ mice seemed to have a greater percentage of responding cells, but a lower U_{osm}, suggesting a lower water permeability per IMP cluster than VII +/+ Normal mice. It was therefore possible that the makeup of clusters was different between the genotypes. Hence, the second question of the present study:

II. What is the nature of IMPs within clusters of responding cells of the mouse collecting duct system?

1.3.3 Cytoplasmic Vesicles in NDI Mice -- While similarities have been observed in the hydroosmotic mechanism of anurans and the urinary concentrating mechanisms of mammals, there are obvious differences between principal cells of the mammalian collecting duct system and the granular cells of the toad bladder. For example, the principal cells, which are sensitive to neurohypophysial hormones, do not have the granules that are present in the analogous toad bladder granular cells. Nor do the principal cells appear to have the tubular IMP-containing "aggrephores" characteristic of the toad bladder granular cells (Brown, unpublished observation). The principal cells in the collecting duct

system of normal mice do, however, have numerous, rounded, cytoplasmic vesicles, which could conceivably subserve a function similar to that of the aggrephores of toad bladder cells.

Unfortunately, unlike the IMPs of the clusters in vesicles or apical membrane surfaces of toad bladder, the IMPs of clusters in mouse or rat collecting duct luminal membranes do not have an easily recognizable, highly characteristic, between-particle morphology (63). Further, in mammals there are no morphological features that distinguish IMPs observed in membranes of the cytoplasmic vesicles from those observed in luminal membranes -- although IMPs certainly are present in both (Brown, unpublished observations).

Although there are some differences between the anuran and mammalian systems, the many similarities of these two systems suggest that a "shuttle" mechanism might exist also within the principal cells of mice. Therefore, if DI +/+ Severe mice show a deficiency of IMP clusters (Question I above), might that deficiency be related to a lack or dearth of cytoplasmic vesicles (analogous to aggrephores found in toad bladder)? Hence, the third question of the present study:

III. What is the status of cytoplasmic vesicles in principal cells of the mouse collecting duct system?

1.4 Rationale of Approach

1.4.1 IMP Clusters -- Fortified by the data shown in Figure 4, this investigation assumed that the intensity of the clustering response reflects the water permeability of the collecting duct system. If this assumption is correct, then DI +/+ Severe mice, in which the presence of an osmotic disequilibrium between collecting duct fluid and the surrounding interstitium (Fig. 6, p. 32) suggests a defect in water permeability,

should have few, if any, IMP clusters. In contrast, VII +/+ Normal mice or CBA Os/+ mice, in which urinary and interstitial osmolality apparently are equal, should have many IMP clusters; and DI +/+ Nonsevere mice, with a possible small degree of osmotic disequilibration (Fig. 6), might have an intermediate number of IMP clusters.

1.4.2 Characteristics of IMP Clusters -- There are many cellular mechanisms which influence the final concentration of urine (see Introduction). As mentioned in 1.3.2, previous studies in toad bladder (23,77) demonstrated that the IMP clustering response in anurans was apparently one of increased density of IMP clusters rather than increased number of intramembranous particles within each cluster. However, preliminary experiments in the present study suggested that the principal cells of CBA Os/+ mice had a greater density of IMP clusters but a lower U_{osm} than did VII +/+ Normal mice. Such a discrepancy could be the result of having osmotic water flow inappropriate to the frequency of IMP clusters, a condition secondary to these mice having small kidneys (111) or foreshortened nephrons (85) [see Section 1.2.2.3]. However, in the context of the present research, it could also result from some difference in IMPs within clusters. If the latter is the case, one might expect the IMPs of CBA Os/+ mice to be different from those of the other strains, in respect to certain characteristics such as size of the particles or number of the particles per cluster.

1.4.3 Cytoplasmic Vesicles -- If the frequency of IMP clusters in the principal cells of mouse collecting ducts is somehow related -- either causally or consequentially -- to the number of cytoplasmic vesicles in the same cells, then: animals found to have few or no IMP clusters (presumably the DI +/+ Severe mice) might have a diminished or absent

cytoplasic vesicle pool; animals with the greatest number of IMP clusters (presumably the VII +/+ Normal mice) should have the largest cytoplasmic vesicle pool; and animals with an intermediate number of IMP clusters (presumably the DI +/+ Nonsevere mice) should have an intermediate number of cytoplasmic vesicles.

2 MATERIALS AND METHODS

2.1 Animals

The various strains of mice that were used in the present study are listed in Table 1. More detailed descriptions of the characteristics of these mice may be found in the Introduction. It should be noted that CBA +/+ mice, a more popular model for the "normal" or control mouse than the VII +/+ mice, were substituted for the VII +/+ strain when examining the effects of the Os gene. The substitution was required due to inability to successfully breed VII +/+ animals. However, the VII +/+ mice and CBA +/+ mice have very similar urinary concentrating abilities.

TABLE 1. Experimental Mice

Mouse Strain	Description	H_2O Permeability of Collecting Duct System	Reported Osmolality[¶] (Mean±SEM) U_{osm}	Papillary
VII +/+	normal; control	no defect	2764 ±232	2771 ±246
CBA Os/+[Φ]	chronic renal failure	no defect	2043 ±95[†] (2141 ±138[▽]	-- 2065 ±312)[▽]
DI +/+ Nonsevere	mild urinary concentrating defect	?partially impaired?	1406 ±77	1561 ±114
DI +/+ Severe	analogue of human nephrogenic DI; hypotonic urine	severely impaired	149 ±19	740 ±85

¶ = Data extracted from Kettyle and Valtin (85). Φ = CBA Normal mice in which Os gene was introduced; the present study assumed that these mice have the same concentrating defect as VII Os/+ described in (85). † = mean value measured in the present study. No data are available for papillary osmolality in the CBA Os/+ mice. ▽ = values from VII Os/+ mice from (85) for comparison with CBA Os/+ mice.

All of the mice were housed in standard plastic cages in an individual room at the Animal Research Facility (ARF) of Dartmouth

Medical School, which provided 12-hour periods of artificial light from 0600 to 1800. They were fed a standard lab chow (Charles River RMH 3000, Agway, Syracuse, NY) ad lib and had free access to tap water.

Urine samples were taken from the mice in this same room and were most easily obtained between the hours of 2000 and 2300, presumably because mice are nocturnal animals. The mice often voided spontaneously when placed on a clean 10 x 15 cm piece of parafilm. Alternatively, urination was induced by abdominal compression. The urine samples were stored at $4^{o}C$, capped, in 250 microliter polypropylene microfuge tubes (Fisher Diagnostics, Orangeburg, NY), until urine osmolality (U_{osm}) was determined. Urine osmolality was measured in duplicate on a Wescor 5100C Vapor Pressure Osmometer (Logan, UT). Because the onset of severe nephrogenic diabetes insipidus is age-dependent within the DI +/+ Severe genotype (Fig. 5)(167), animals were not classified as DI +/+ Severe unless three successive samples of their urine were shown to be hypotonic to plasma.

DI +/+ Severe mice not only have hypotonic urine, but also elevated levels of endogenous vasopressin (150)[DI +/+ Severe mice 17.2 $\pm$3.9 picograms (pg) vasopressin per ml blood; VII +/+ Normal mice 5.0 $\pm$1.4 pg/ml]. However, in order to minimize the possibility that a lack of vasopressin could be the reason for their failure to increase water permeability of the collecting duct, some of these mice were given intraperitoneal (i.p.) injections of 1-desamino-8-D-arginine vasopressin (DDAVP)(Minipress, Ferring, Sweden). Three DI +/+ Severe mice received bolus i.p. injections of 2,500 nanograms (ng) per kg body weight (approx. 6000 x normal) for 3 days and two received 250 ng per kg body weight (approx. 600 x normal) for 4 days. The three given the higher dose were sacrificed approximately 24 hours after the last injection; the two given the lower dose were sacrificed on the day of the fourth injection, 2 and

3 hours, respectively, after the injection. The biological activity of the DDAVP was assessed by injecting two homozygous Brattleboro rats with 30 ng per kg body weight. Mean U_{osm} in these rats rose from 174 to 913 mOsm/kg H_2O in four hours.

2.2 Quantification of IMP Clusters

2.2.1 Tissue Preparation -- The animals were brought to the laboratory from the Animal Research Facility only a few at a time to minimize the time spent in unfamiliar surroundings. An acute rise in urinary concentrating ability observed in preliminary experiments was thought to be due to stress accompanying these environmental changes. Spot urine samples were collected from each mouse by the methods described above, or immediately after sacrifice when loss of voluntary sphincter control released urine stored in the bladder.

The animals were sacrificed by cervical dislocation. After a mid-abdominal incision was made, the kidneys were removed in random order. The renal artery and vein of the first kidney to be removed were clamped with a hemostat in order to minimize blood loss prior to the excision of the contralateral kidney. After removal, each kidney was cut eccentrically in the sagittal plane, about 3/8 : 5/8, so that the whole papilla was retained in the 5/8 portion. Then the tissues of the cortex and outer medulla were rapidly cut away from the 5/8 portion, leaving just the inner medulla. The final 2 mm of the papilla were discarded because Lacy (92) has demonstrated that this portion shows a diminished IMP clustering response to vasopressin, at least in rats. The unsectioned papillae were then fixed in 2.5% glutaraldehyde/0.1M sodium cacodylate (Electron Microscopy Sciences, Fort Washington, PA) for 4 hours. After 4 hours, the papillae were washed three times for 5 minutes in 0.1M sodium cacodylate, and stored in that same buffer. The containers were then

coded so that subsequent processing and analysis for IMP clusters would be performed without prior knowledge of the experimental history of the tissue.

When experiments early in this investigation suggested that the collecting ducts of DI +/+ Severe mice did not have the IMP clustering response (see Results), an additional 8 DI +/+ Severe animals were examined using a slightly different protocol. An attempt was made to induce IMP clustering in vitro by incubating the papillae from these mice with agonists in various combinations, in Dulbecco's Modified Minimum Essential Medium (KC Biological, Lenexa, KS). The papillae were placed with the various treatments in an incubator with 95% room air, 5% CO_2, saturated with H_2O, at 37°C for 30 minutes before being fixed in the glutaraldehyde/cacodylate buffer. The protocol was otherwise identical. Because of experimental difficulties (see Appendix), only control tissues for these treatments are included in the present study. These tissues were also coded and analyzed for the presence or absence of IMP clusters.

2.2.2 Freeze Fracture

2.2.2.1 Tissue Processing -- Prior to the fracturing process, the samples were put into 0.1M sodium cacodylate which contained 30% glycerol. All tissues remained a minimum of 2 hours in this solution to allow infiltration of this cryoprotectant to the interior of each papilla. The papillae were then prepared for sectioning on a Model 501 vibratome (Oxford Instruments, San Mateo, CA). They were removed from the cryoprotectant solution, blotted, and affixed to the glass stage of the vibratome with either Superglue (Loctite Corp., Newington, CT), or Krazy Glue (Krazy Glue Inc., Itasca, IL); care was taken to use as little of the cyanolate glue as possible and still get firm adhesion, since the glue interferes with subsequent steps of the processing. Using a vibratome

speed of 3 and an amplitude of 7, sections were cut initially 20 micrometers thick. These relatively thin sections (for non-embedded tissues) were used because it is a general rule that thinner sections result in more rapid freezing and less subsequent formation of ice within the tissue. In the later stages of these experiments, however, the thickness of sections was increased to 50 to 60 micrometers, when it was discovered that the thicker sections did not enhance ice formation but did increase the incidence of successful fractures.

2.2.2.2 Freezing the Tissue -- Replicas of the fractured tissue surfaces were prepared according to the procedures of Moor et al (105), but with slight modification. Cylindrical, 24 karat gold supports, approximately 1.5 mm in diameter, were cleaned thoroughly by sonification. A small drop of Vinol, a polyvinyl alcohol solution consisting of 20 grams Vinol dissolved in 100 ml phosphate-buffered saline containing 20% glycerol, was placed on each support with a fine forceps. According to Brown (15), in addition to enhancing the fracturing properties of the tissue, the Vinol serves as an additional cryoprotectant and helps attach the tissue to the support. One section of papilla was placed on the Vinol and flattened to the support surface with the forceps, and a small drop of cacodylate/glycerol was left on the surface of the tissue. Two other samples were prepared identically. Procedures from this point to the immersion of the supports into liquid nitrogen were carried out as rapidly as possible, to prevent evaporation of the cacodylate/glycerol and consequent changes in viscosity and osmolality, which would affect the tissue surface and hence the ultimate fidelity of the replica.

After the above preparation, gaseous Freon 22 was slowly added to a cylindrical, chrome-plated, copper bowl, which was suspended in a liquid nitrogen-filled thermos. As the Freon 22 liquefied in the bowl, the

Freon nozzle was "swirled" to prevent adherence to the bottom of the bowl. When almost full, the nozzle was simultaneously turned off and quickly removed from the liquid Freon to prevent either freezing the Freon in the nozzle (turned off too early), or blowing the liquid Freon 22 from the bowl (turned off too late). Forceps angled 45°, which had not been cooled previously, were then inverted and used to pick up one of the supports. Then the forceps and support were re-inverted and immersed in the Freon for about 10 seconds. There could be no hesitation over the Freon, since the very cold air above the Freon could begin a freezing process much slower than the instantaneous freezing process required to prevent the formation of internal ice crystals. The sample had to be inverted so that the tissue hit the Freon before the body of the support did, and froze immediately and completely. With a similar, rapid, up-down motion, the supports were withdrawn and placed into a container of liquid nitrogen, where any Freon remaining on the support or tissue solidified. The rapid motion facilitated removal by inertia of any excess liquid Freon 22 and protected the sample from thawing. The other two samples were treated identically, each with a different, room-temperature forceps. The samples were then brought into juxtaposition with the Balzers 301 Freeze Etch unit, equipped to provide a high vacuum with a DUO 35 rotary vacuum pump and a DIFF 900 diffusion vacuum pump (Balzers High Vacuum Corp., Liechtenstein).

2.2.2.3 Fracturing the Tissue -- The stage, but not the knife, of the Balzers 301 was initially cooled with the GA-1 to -170°C, and the bell was put under high vacuum to remove any liquid or vapor residues in the bell. To introduce the samples, the vacuum was released and the supports were transferred very rapidly from the liquid nitrogen to the stage with a forceps whose tip had been cooled in the liquid nitrogen. Any previ-

ously solidified Freon 22 was allowed to reliquefy on the stage, which had now begun to warm up slightly. Then the support was slid under the clamp on the stage and the clamp secured. The remaining two supports were transferred in a similar manner, insuring that the clamp was refastened for each support so that thermal conductivity was maintained and no thawing of the tissue could take place. Then the GA-1 was set to adjust stage temperature to -100°C and to begin cooling of the knife to -170°C. When the knife reached -170°C, it was positioned as close as possible in the vertical plane to the surface of the tissues on the supports, and the bell was pumped down to 5×10^{-7} Torr or less. Any ice on the surface of the tissues sublimed in the vacuum and recrystallized on the cooler knife. Therefore, this procedure cleaned the ice from the surface of the tissue, and assisted greatly in achieving a high vacuum, usually within an hour and a half.

When the majority of ice had disappeared from the surface of the tissues, the samples were ready for fracturing. A degreased, single-edged razor blade, inserted into the knife holder during the clean-up operation after the previous fracture, was drawn over the sample slowly, slightly shaving the surface in successive repetitions, until reaching the tissue in the center of the frozen glycerol on the supports. The blade was moved slowly so that the tissue surface would be fractured due to stress from its edge rather than from cutting. The slow movement also helped to prevent any heat from developing from friction during the cut, which would tend to thaw the tissue. The depth of the tissue within the frozen cacodylate/glycerol could be roughly estimated by the frozen "outline" of the slice of tissue itself, and by the fact that, as it was being fractured, the tissue surface appeared to be more translucent than the more transparent frozen buffer. The samples were fractured in order, and care was taken that the cutter blade was never drawn back over the

newly fractured tissue surfaces, lest ice particles fall onto the surface and distort the resulting replica. Application of platinum and carbon onto these newly fractured surfaces to form the replica was controlled by the Balzers electron beam evaporation device (EVM-052).

2.2.2.4 Application of Platinum and Carbon -- Platinum was evaporated onto the samples from a gun positioned 45° to the tissue surface (Fig. 3). Using the EVM-052, filament 1 (platinum) was selected, the voltage was set to 1500 volts (v), and the current to 60 milliamps (mA). The thickness of both the platinum and carbon accumulating on the tissue surface was measured by a quartz thin monitor (QSG 201), which gave an audio tone and a meter indication of the amount of material simultaneously accumulating on its own quartz surface. With a range of 1.0 set on the QSG 201, evaporation was continued until 4.0 on the meter. Immediately, the EVM-052 was reset; filament 2, a carbon gun aimed 90° to the tissue surface, was selected; the voltage was set to 1100 v; and the current was set to 180 mA. With a range of 0.3 on the QSG 201, evaporation was continued until 8.5 on the meter. This evaporation, however, had an initial period of false evaporation as the filament heated the carbon electrode, but it stopped abruptly after a few seconds. The true evaporation took place shortly thereafter, without the operator having to adjust any of the controls. The audio signal was used to qualitatively monitor the progress of the evaporation, as the frequency of the signal increased in direct proportion to the amount of accumulating material. Both evaporations were stopped by interposing the knife between the evaporation guns and the tissue surfaces to stop the accumulation of material before the EVM-052 was shut off or reset. When both evaporations were complete, the vacuum was discontinued, and the supports were removed and transported in a petri dish for recovery of the

replica.

2.2.2.5 Processing the Replicas

2.2.2.5.1 Removing Tissue from the Replica -- Since abrupt changes in surface tension can fragment the replicas as they separate from the supports, the latter were lowered very slowly with forceps into porcelain spotting wells which contained a hypochlorite solution at room temperature. Sometimes the replicas spontaneously lifted off the support surface and floated on the surface of the hypochlorite. More often, however, 15 to 30 minutes of immersion in the hypochlorite was required before the replicas separated from the supports. Usually they remained below the surface. In either case, the replicas were left in this solution for about 3 hours, with one change of solution, to remove tissue, which adhered to the platinum and carbon replica. If the replicas were left in the hypochlorite much longer than 3 hours, they became too clean, and, if portions of the replica folded over on themselves, they stuck together permanently.

2.2.2.5.2 Degreasing and Recovery of the Replicas -- The tip of a 5 and 3/4 inch borosilicate glass Pasteur pipette was heated until it melted into an attached, rounded drop, and then it was allowed to solidify. This device was used to recover the replicas from the hypochlorite solution. The solution was stirred carefully with this device to vortex the replica up from the bottom of the well without fragmenting the replica. The rounded-drop end of the glass device was then inserted under the now suspended replica and used to lift it from the solution. The replica was transferred very rapidly into another porcelain well containing 2 parts chloroform to 1 part methanol. In the new well, the replica was stirred gently for a moment with the device while any

remaining drops of hypochlorite dispersed, so that any differences between solutions in surface tension or vapor pressure did not cause the replica to adhere to the sides of the well. Additionally, the glass recovery device was wiped dry when going from solution to solution because residual droplets could cause turbulence severe enough to fragment the replica.

The replicas remained for at least 15 minutes in the chloroform-/methanol for degreasing. Care was taken that this very volatile mixture did not evaporate so much as to allow the replica to stick to the wall of the well and dry, or the replica would have been lost. In fresh chloroform/methanol, evaporative forces would usually cause a rolling turbulence in the well. The replica would be carried around in this wave of turbulence. A Pasteur pipette was filled with distilled water, which was introduced a drop at a time into the chloroform/methanol. With practice, the rolling movement of the replica could be timed, and a water drop introduced to the surface at the edge of the well just as the replica was passing close by. The immiscible nature of the two fluids created a slightly more violent turbulence. Successful recovery depended upon trapping the replica in the interface of the water and chloroform/methanol, a procedure more easily done while observing the interface with a dissecting microscope at 20 power. When the replica suddenly flattened out, all the fluid was quickly withdrawn with the pipette, using care not to aspirate the replica itself. The replica would then lie flat on the porcelain surface. After a few moments to allow residual chloroform/methanol to evaporate, the well was filled slowly with distilled water and the flattened replica usually floated up from the well wall onto the surface of the water. However, if there was any residual chloroform/methanol left on or near the replica, the addition of water often caused the replica to fragment violently. If the

previous steps were accomplished with sufficient care, the free-floating replicas were then recovered from the surface of the water on forceps-held, parlodion-coated, copper grids, and subjected to analysis by electron microscopy.

2.2.3 Scoring Replicas

2.2.3.1 Morphology -- Replicas were examined for IMP clusters by electron microscopy (Philips EM300). All freeze fracture replicas were examined in their entirety for the presence of apical membrane belonging to principal cells of collecting ducts. The morphological markers described by Orci et al (115) were used to differentiate principal cells

Figure 7. Principal cells of the mouse collecting duct. The multiple-stranded structures in the uppper right of the micrograph are tight junctions typical of these cells (115). x24,000.

from other cells present in the replicas. For example, since within the papilla only principal cells have very complex, multiple-stranded, tight junctions, such as those shown in Fig. 7, the presence of this marker was a positive identification. If the tight junction was not visible, the presence of several other features in combination sufficed. The microvilli of principal cells (Fig. 8) are more irregular in shape and appear to have less distinct edges than those of cells from the thin ascending limb of Henle's loop. The microvilli of cells from the lower part of the descending thin limb of Henle's loop (Fig. 9), appear as almost perfect hemispheres. Upper parts of descending thin limbs of Henle's loop have microvilli more similar to those of principal cells, but they have a

Figure 8. Freeze fracture luminal membrane surface of principal cells from mouse collecting duct. Note irregularly-shaped microvilli over most of the cell surface. Compare with Fig. 9. x24,000.

Figure 9. Freeze fracture luminal membrane surface of cell from descending thin limb of Henle's loop. Microvilli appear as almost perfect hemispheres. Compare to Fig. 8. x25,000.

great many more intramembranous particles per square micrometer, which do not aggregate into clusters. Additionally, principal cells are visibly thicker than both types of descending limb cells. Finally, cells from the descending thin limb of Henle and principal cells are, of course, located in different tubules within the nephron, so that if positive identification of a single principal cell can be made and its adjoining lumen traced, the other cells around the same lumen are very likely to be principal cells also. Dark, or mitochondria-rich, cells can be differentiated from principal cells easily because their apical surface and/or cytoplasmic vesicles contain rod-shaped intramembranous particles (115).

All principal cells with apical surface sufficient to fill the

photographic frame of the microscope at a magnification of x14,000 could be scored as negative or positive for IMP clusters with the knowledge of certain morphological characteristics. On the E face or leaflet, microvilli appear as depressions and IMP clusters as particles on slight mounds. On the P face, the microvilli appear as hills, and the IMP clusters in slight depressions. Although we scored a principal cell as positive if it had only one IMP cluster, most positive cells had many clusters, and were easily distinguished qualitatively from those that did not have the clusters (see Figs. 10 and 11, pp. 59 and 61).

2.2.3.2 Density of Clusters per Cell -- Initial qualitative observations suggested that IMP clusters in principal cells of these mice were homogeneously distributed in those cells exhibiting the clustering phenomenon. Consequently, the central portion of the apical membrane of each cell which had clusters was photographed at a magnification of x22,000 for quantification of numbers of IMP clusters per square micrometer of apical membrane. Positive prints were made of these exposures at a final magnification of x66,000. These photographs were then visually scored for number of IMP clusters per photograph, and a Tektronix 4953 graphic tablet (Tektronix International AG, Zug, Switzerland) was used to quantify the total membrane surface area in the same photograph.

2.3 Characteristics of IMP Clusters

2.3.1 Density of IMPs per Cluster -- The number of individual particles within each IMP cluster on E faces of these same prints was also counted visually. The E face was used because individual particles within the clusters tend to be less distinct on the P face. A total of 169 clusters from VII +/+ Normal mice, 139 clusters from DI +/+ Nonsevere

mice, and 209 clusters from CBA Os/+ mice were thus evaluated.

2.4 Quantification of Vesicles

2.4.1 Tissue Preparation -- Some of the sections of papillae cut by vibratome were also utilized for thin section transmission electron microscopy. The sections, which had been preserved in 0.1 M cacodylate, were first post-fixed in 1% OsO_4 for 1 hour. Then they were washed 3 times for 5 minutes in the cacodylate buffer. Next, the tissues were dehydrated in graded ethanols and embedded in Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA) according to the method of Simionescu et al (142). Standard procedures were then used to make semithin and ultra-thin sections.

2.4.2 Scoring of Thin Sections -- Ultrathin sections were examined by electron microscopy (Philips EM300) for the presence of vesicles without a bristle coat near the apical surface. These vesicles would be the ones most likely to have been formed recently during the process of endocytosis, and therefore, theoretically, could represent "aggrephore-type" vesicles. Principal cells were easily differentiated from loop of Henle cells because the principal cells have a thicker cytoplasm and form lumens that are larger and more irregularly shaped than lumens formed by loop of Henle cells. When possible, the electron microscope grids were maneuvered so that the image of the apical membrane diagonally bisected the photographic frame, thereby maximizing the surface area available for analysis. Photographs at a magnification of x8,000 were taken of randomly selected cells from 3 randomly selected mice of each genotype. Positive prints of these exposures at a final magnification of x24,000 were examined visually for the presence of rounded, membrane-bound vesicles without an obvious bristle coat within 1 micrometer of the

apical surface (see Figs. 14 and 15, p. 68). To standardize vesicle counts for comparison among strains of mice, the counts were reported as vesicles per micrometer of apical surface examined. Consequently, either the Tektronix 4953 graphic tablet, or a 2000 Series Digitizer (Summagraphics, Fairfield, CT) coupled to a VAX 11/7800 computer (Digital Equipment, Maynard, MA), was used to quantify the apical membrane surface in micrometers. Two different systems were used because of varying geographic locations of the equipment (i.e., Geneva, Switzerland and Hanover, NH, U.S.A). However, measurements from the two systems were compared against each other to insure accuracy between the systems. Two identical prints were analyzed for membrane surface using each system. Identical values measured by each system confirmed that the systems could be used interchangeably.

2.5 Statistics

All statistics were performed according to the methods outlined by Zar (175). For data approximating a normal distribution, analyses included standard descriptive statistics (mean, range, etc.), regression analysis by least squares, one way analysis of variance (ANOVA), and Newman Kuels Multiple Range Test for unequal sample sizes.

Data which included values for the proportion of responding cells were presumed to be nonparametric because of the numerous 0.00% responses recorded for the DI +/+ Severe mice. If the data which were presumed to be nonparametric were, in fact, from a normal population, the nonparametric statistical tests would have had 95% the probability of detecting statistical differences (rejection of the null hypothesis) as compared to ANOVA and the Newman-Kuels tests (175). Therefore, utilizing the more conservative approach, analyses which included data from the DI +/+ Severe mice were accomplished using the nonparametric Kruskal-Wallis test

if there were more than two groups in the comparison, or the nonparametric Mann-Whitney test if there were only two groups in the comparison. If either of these tests detected statistical differences between groups, two additional tests were performed to determine which groups were different. If sample sizes of the groups were equal, the multiple comparison rank test described by Zar was performed (175). If sample sizes of the groups were different, then a new procedure called the Kruskal-Wallis All Subset procedure for Multiple Comparisons, described by Campbell and Skillings (21), was utilized.

As previously described, major parts of the present study were performed to determine if any association existed between the independent variables U_{osm} and IMP cluster frequency, or between the independent variables IMP cluster frequency and occurrence of cytoplasmic vesicles. However, it was not proper to compare these two independent variables by the calculation of correlation coefficient (assumes normal distribution) because of the numerous 0.00% IMP cluster frequency responses of the DI +/+ Severe mice (a significant deviation from normality). Consequently, the comparisons of IMP cluster frequency and U_{osm}, and IMP cluster frequency and cytoplasmic vesicles were accomplished by the calculation of the nonparametic Spearman's Rho Rank Correlation coefficient, r_s.

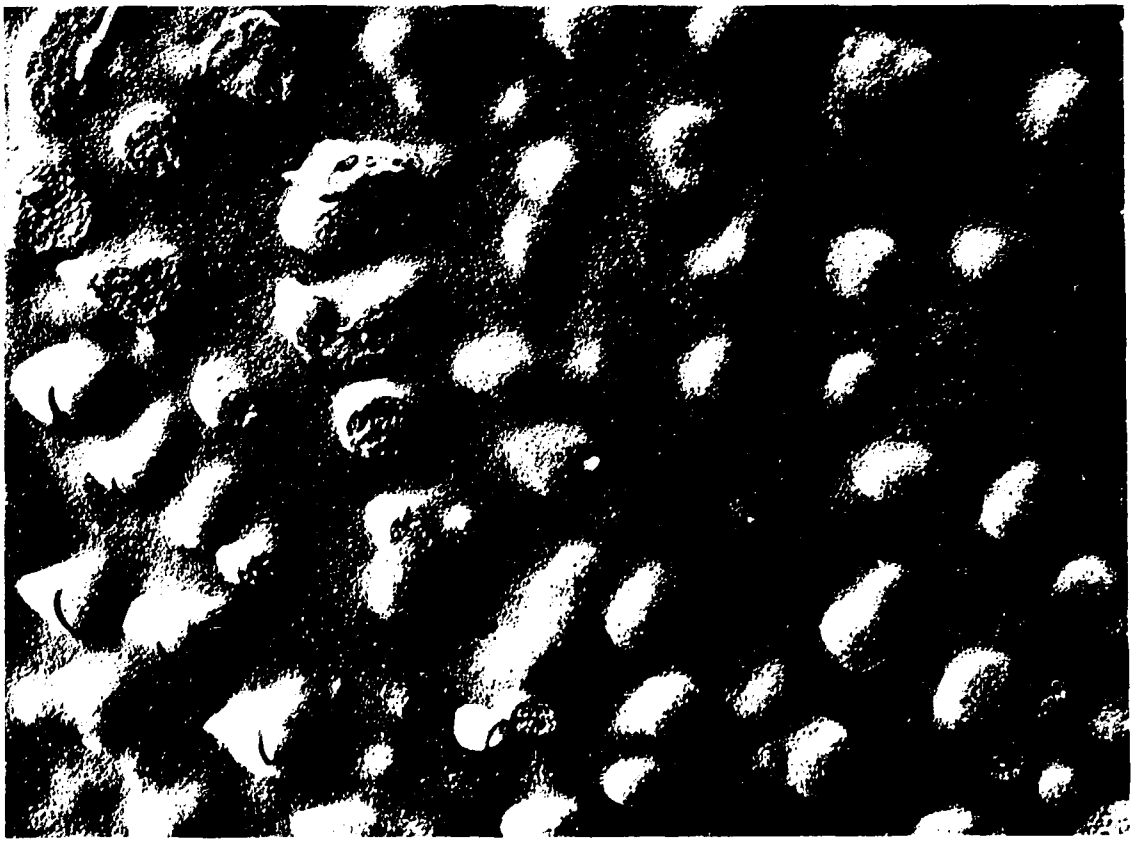
Significance for all parametric and nonparametric statistical operations was set at $p \leq 0.05$. The exact P values derived in each operation are reported in the text or in the legends of the graphs and tables.

3 RESULTS

3.1 IMP Clusters

3.1.1 Cluster Frequency -- Quantitative data concerning IMP clusters are presented in Tables 2 through 5, pp. 60, 62, 65, and 66, respectively. No IMP clusters were found in the first six DI +/+ Severe mice (Fig. 10), even when the mice had been pretreated with exogenous DDAVP. Additional DI +/+ Severe mice were examined to rule out the possibility that IMP clusters had been fortuitously overlooked. Papillae which were fixed immediately after excision from two additional DI +/+ Severe mice, prepared according to the original protocol (see Materials

Figure 10. Freeze fracture micrograph of luminal membrane of a principal cell from the collecting duct of a DI +/+ Severe mouse. This cell does not demonstrate the IMP clustering response. x54,000.



and Methods), as well as papillae incubated for 30 minutes in vitro after excision, from 8 other DI +/+ Severe mice, all lacked IMP clusters. All of these DI +/+ Severe mice had very low urine osmolalities (Table 2, p. 59), mean (n=16) U_{osm} = 180 $\pm$22 mOsm/kg H_2O.

In contrast, all VII +/+ Normal mice, all DI +/+ Nonsevere mice, and all CBA Os/+ mice had IMP clusters to varying degrees (Fig. 11) and were statistically different from each other ($P<0.02$). The CBA Os/+ animals had the highest frequency of responding cells -- 74 cells positive (i.e., containing one or more clusters) of the 86 cells examined, for an 84% response. VII +/+ Normal mice demonstrated a slightly lower, positive response -- 126 positive cells of the 237 cells examined, for a 52% re-

Figure 11. Freeze fracture micrograph of luminal membrane of principal cell from the collecting duct of a VII +/+ Normal mouse. This cell demonstrates the typical IMP clustering response. x54,000. Inset: typical intramembranous particle cluster. x90,000.

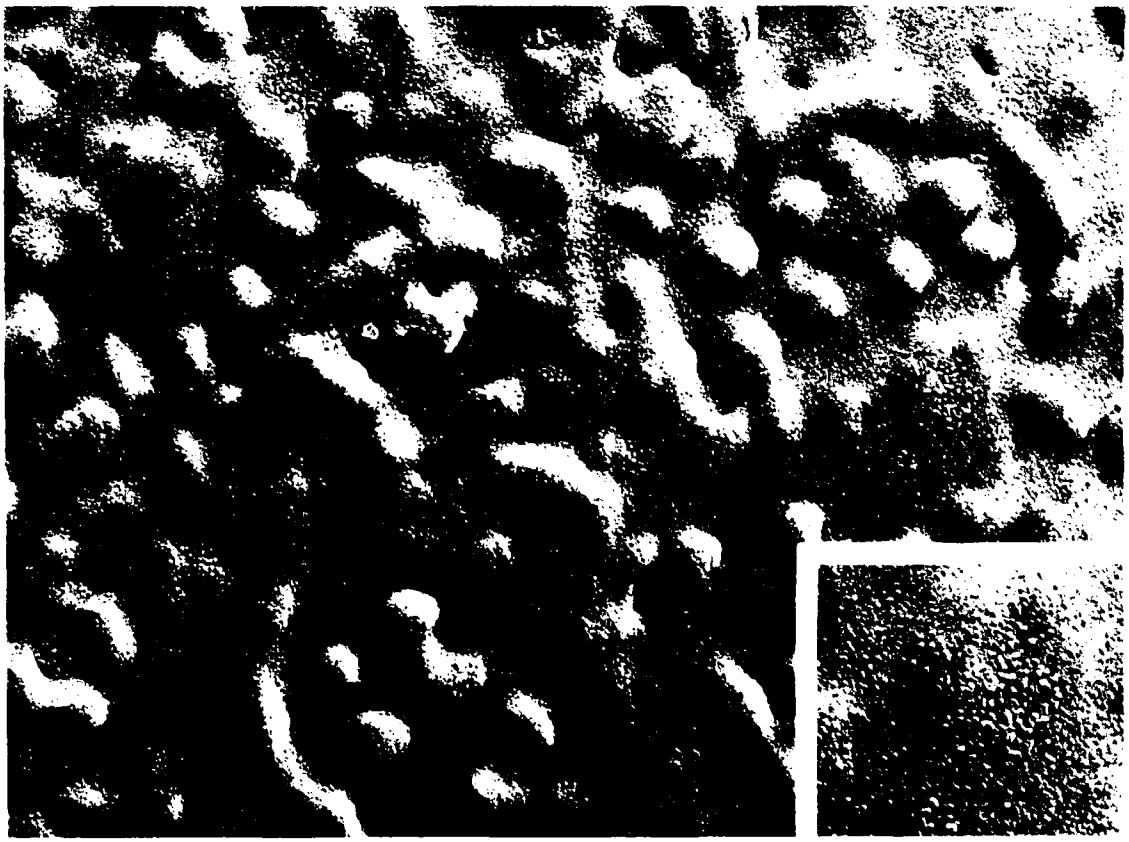


TABLE 2. Urinary Osmolalities and IMP Clustering Response of Principal Cells

Mouse Genotype	Mouse ID Number	U_{osm}	+ Cells $^{\Phi}$	- Cells	+ Cells/Total (%)
VII +/+ Normal	349.2E	1673	11	19	37
	349.2F	*	32	23	58
	349.2G	2016	18	22	45
	366.1B	2382	17	16	52
	377.1B	2812	15	18	45
	366.1A	2289	33	13	72
$\bar{x}(\pm SE)$		2234 $\pm$190			52 $\pm$5 $^{\nabla}$
CBA Os/+	7.1A	1862	27	3	90
	7.1B	2231	27	4	87
	7.1C	1898	12	1	92
	8.3A	1862	8	4	67
$\bar{x}(\pm SE)$		2043 $\pm$95			84 $\pm$6 $^{\nabla}$
DI +/+ Nonsevere	712.3C	1116	5	10	33
	715.1C	1542	7	14	33
	712.4B	947	8	35	19
	752.4E	1114	12	16	43
	754.1E	1018	14	38	27
	752.4D	1060	13	16	45
$\bar{x}(\pm SE)$		1133 $\pm$86 $^{\delta}$			33 $\pm$4 $^{\nabla}$
DI +/+ Severe	718.1F	213	0	30	0
	718.1E	462	0	33	0
	718.1C	224	0	27	0
	731.3I †	180	0	20	0
	731.3H †	204	0	16	0
	735.1B †	189	0	35	0
	744.3C ¶	77	0	34	0
	744.3B ¶	73	0	40	0
$\bar{x}(\pm SE)$		203 $\pm$43 $^{\delta}$			0 $\pm$0 $^{\nabla}$
DI +/+ Severe (Incubated in vitro)	753.3E	198	0	23	0
	748.1F	112	0	12	0
	744.4B	132	0	16	0
	748.2C	127	0	11	0
	743.2D	199	0	2 $^{\alpha}$	0
	753.1D	131	0	3 $^{\alpha}$	0
	744.4C	214	0	22	0
	748.1G	148	0	2 $^{\alpha}$	0
$\bar{x}(\pm SE)$		158 $\pm$40 $^{\delta}$			0 $\pm$0 $^{\nabla}$

*Insufficient urine for analysis. $^{\alpha}$ =small tissue samples. U_{osm} is in mOsm/kg H_2O. $^{\Phi}$ = cells have at least one IMP cluster; - cells showed no clusters. † =received 250 ng.kg body weight^{-1}.day^{-1} DDAVP s.c. for 3 days; sacrificed 24 hours after last injection. ¶ =received 2500 ng.kg body weight^{-1}.day^{-1} DDAVP s.c. for 3 days; sacrificed 2 hours after last injection. $^{\nabla}$ =different from other genotypes ($P<0.02$). $^{\delta}$ =U_{osm} significantly different from that of VII +/+ Normal mice ($P<0.001$).

sponse. The DI +/+ Nonsevere mice had 59 cells with IMP clusters of the 188 cells examined, a 33% response.

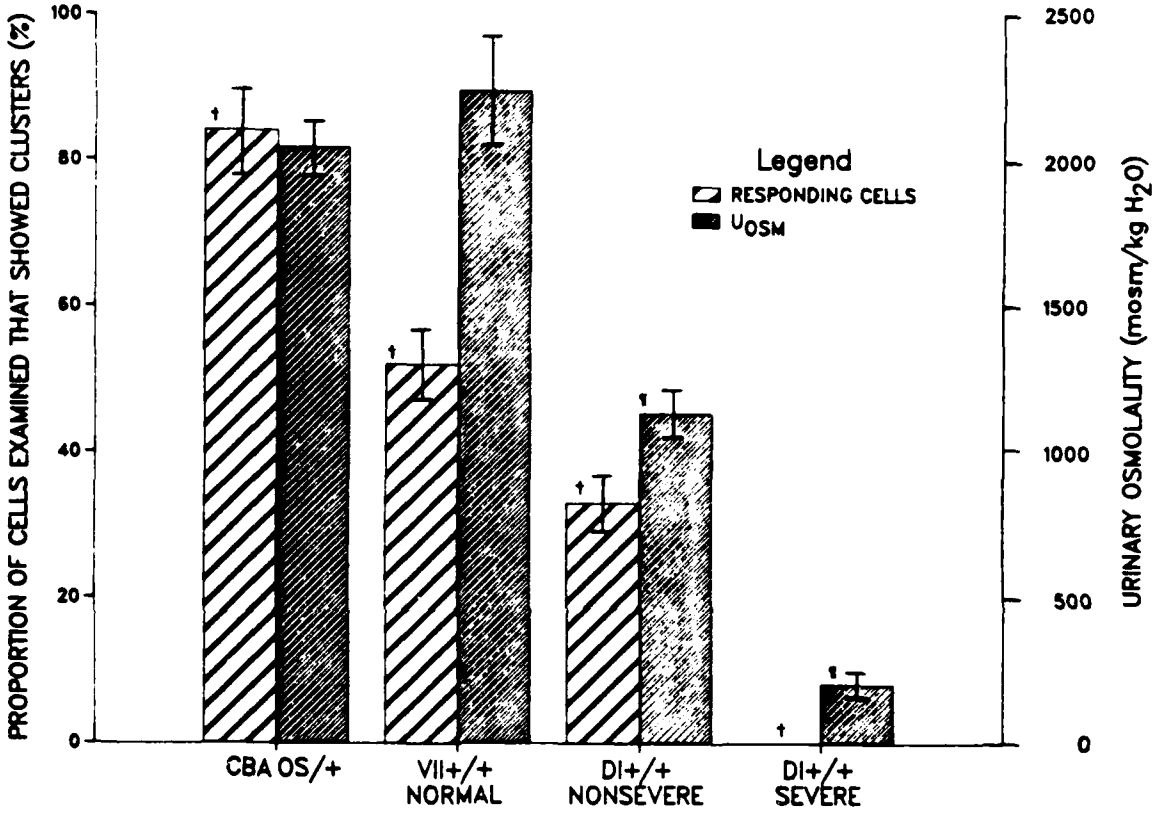
3.1.2 Cluster Density -- While cluster frequencies between the mouse strains were statistically different, the density of clusters per square micrometer of cell surface examined was not (P=0.05). VII +/+ Normal mice had 1.16 ±0.07 clusters per square micrometer; DI +/+ Nonsevere mice had 1.13 ±0.11 clusters per square micrometer; and CBA Os/+ mice had 1.08 ±0.07 IMPs per square micrometer (Table 3).

Table 3. Cluster Density

	-Mouse Genotype-		
	VII +/+ Normal	CBA Os/+	DI +/+ Nonsevere
Mean IMP Clusters (±SEM) per square micrometer of surface examined	1.16 ±0.07	1.08 ±0.07	1.13 ±0.11

3.1.3 Cluster Frequency and Urine Osmolality -- The results and statistical analyses from the first four groups of animals in Table 2 are shown in Fig. 12. The strong correlation between proportion of cells with clusters and U_{osm} suggested by the graph was confirmed statistically: Spearman rank correlation coefficient, r_s=0.891 (P<.001). [Although DI +/+ Severe data led to a presumption of a non-normal distribution, correlation coefficient, r, a statistic computed using data from a normal distribution, was computed for comparison with r_s. The value computed for r, 0.85 (P<0.001), also suggested that a strong association existed]. Additionally, the Kruskal-Wallis test, followed by the Newman-Kuels Multiple Range Test for unequal sample sizes, revealed that the mean urine osmolalities of DI +/+ Severe mice (203 ±43 mOsm/kg H_2O)

Figure 12. Urine osmolalities and proportion of collecting duct cells examined that showed IMP clusters. All values are mean ±SEM. Spearman rank correlation coefficient, r_s=0.891 (P<.001), between urinary osmolality and % responding cells. ¶ =different from urinary osmolality of VII +/+ Normal mice (P<0.001). † =different from other genotypes (P<0.02).



and DI +/+ Nonsevere mice (1133 ±86 mOsm/kg H_2O) were different from the U_{osm} of VII+/+ Normal mice (2234 ±190 mOsm/kg H_2O) (P<0.001). Mean U_{osm} of the CBA Os/+ mice (2043 ±95 mOsm/kg H_2O) was not different from that of VII +/+ Normal mice (P=0.05). The All Subset procedure for Kruskal-Wallis Multiple Comparisons with uneven sample sizes described by Campbell and Skillings (21) demonstrated that the proportions of responding cells for all genotypes were statistically different from each other (P<0.02).

3.1.4 Homogeneity of Response -- In the mice which had IMP clusters, the clusters were similar to those described previously for rats (19,63,

Figure 13. Freeze fracture micrograph of collecting duct cells from a VII +/+ Normal mouse. The upper cell, divided by cell junction from other cells, has IMP clusters, while the lower cell does not. x34,000.

64), with one exception. Negative cells of any of the mouse genotypes (i.e., Fig. 10, p. 59) did not appear to have the very small clusters found in "poorly responding", or unstimulated cells in the rat (63,64). In the mouse genotypes which showed clusters, there were no apparent differences in the general appearance of the clusters. It was nevertheless possible to find strongly positive cells adjacent to cells with no observable clusters (Fig. 13). The existence of such a heterogeneous response between cells suggested that there might also be a heterogeneity of response within the clusters themselves. Therefore, analyses on the nature of the clustering response were performed.

3.2 Characteristics of IMP Clusters

Particles within each cluster were quantified to determine if there were any differences in the IMP clustering response among the CBA Os/+, DI +/+ Nonsevere, and the VII +/+ Normal mice (Table 4). There were no significant differences between VII +/+ Normal and DI +/+ Nonsevere mice in (a) the mean number of IMPs per cluster, or (b) the size distribution of clusters (Table 5). VII +/+ Normal values for the above characteristics were: (a) 15 to 64, with a mean ($\pm$SE) of 31.6 $\pm$0.77 IMPs per cluster; and (b) 14% <20 IMPs per cluster, 67% 20-40 IMPs per cluster, 19% 41-60 IMPs per cluster. Values for DI +/+ Nonsevere were: (a) 15 to

TABLE 4. Number of Particles per IMP Cluster

Mouse Genotype	Mouse ID Number	U_{osm}	+ Cells Observed	Clusters Counted	$\bar{x}$ Particles per Cluster
VII +/+ Normal	349.2E	1673	11	71	30.6
	349.2G	2016	18	55	33.0
	366.1B	2382	17	17	28.2
	377.1B	2812	15	2	36.0
	366.1A	2289	33	24	33.4
	$\bar{x}$($\pm$SE)	2234 $\pm$190			31.6 $\pm$.77
CBA Os/+	7.1A	1862	27	53	46.3
	7.1B	2231	27	94	37.6 $^{\nabla}$
	7.1C	1898	12	33	53.9
	8.3A	1862	8	29	49.2
	$\bar{x}$($\pm$SE)	2043 $\pm$95			44.0 $\pm$.96 ¶
DI +/+ Nonsevere	712.3C	1116	5	33	33.4
	715.1C	1542	7	47	35.4
	712.4B	947	8	41	35.1
	752.4E	1114	12	15	27.3
	754.1E	1018	14	3	33.7
	$\bar{x}$($\pm$SE)	1147 $\pm$104			33.9 $\pm$.90

The discrepancy between the numbers of positive cells and the numbers of clusters counted is due to the necessity of counting particle density only on E face replicas, which have more distinct particles. + cells observed is the sum of E and P face replicas. $^{\nabla}$ =statistically different from other CBA Os/+ mice (P<.001). ¶ =statistically different from DI +/+ Nonsevere and VII +/+ Normal (P<.001)[Identical results for comparison both with and without aberrant CBA Os/+ Mouse 7.1B].

TABLE 5. Characteristics of Particles within IMP Clusters

Characteristic	VII +/+ Normal	DI +/+ Nonsevere	CBA Os/+
Number of IMPs per cluster (Range)	15-64	15-60	18-81
Mean (±SE)	31.6 ±.77	33.9 ±.90	44.0 ±.96$^{\alpha}$
Size Distribution (IMPs per cluster)			
<20	14%	12%	1%
20-40	67%	66%	45%
41-60	19%	22%	39%
61-81	0%	0%	15%

α =statistically different from VII +/+ Normal and DI +/+ Nonsevere mice ($P<.001$).

60 with a mean(±SE) of 33.9 ±0.90 IMPs per cluster; and (b) 12% <20 IMPs per cluster, 66% 20-40 IMPs per cluster, 22% 41-60 IMPs per cluster. However, the size distribution of clusters for CBA Os/+ mice was markedly different.

Reference to Tables 4 and 5 readily demonstrates that in CBA Os/+ mice, the average number of particles per cluster and distribution of particles in the two highest categories, were increased compared to those of VII +/+ and DI +/+ Non-severe mice. CBA Os/+ mice had 39% of their clusters in the 41 to 60 particles per cluster range, compared to 19% and 22% for VII +/+ and DI +/+ Nonsevere mice, respectively (Table 5). In addition, 15% of the clusters of CBA Os/+ mice had more than 61 particles per cluster, compared to none for either the VII +/+ or DI +/+ Nonsevere mice (Table 5). [The value for average particles per cluster for CBA Os/+ mouse 7.1B was different from the other CBA Os/+ mice. However, average particles per cluster for CBA Os/+ mice was statistically different from VII +/+ Normal mice and DI +/+ Nonsevere mice, both with and without the aberrant data of 7.1B.]

TABLE 6. Frequency of Cytoplasmic Vesicles and IMP Clusters in Representative Mice

Mouse Genotype	Mouse ID #	Number of Cells Examined	Apical Surface (μm)	Number of Vesicles Observed	Vesicles/ micrometer	% Cells with IMP Clusters
VII+/+	366.1B	9	79.6	104	1.30	52
Normal	377.1B	9	90.4	156	1.73	45
	366.1A	10	105.4	154	1.38	72
				$\bar{x}(\pm SE)$	$1.47 \pm .57$	55 ± 7
DI+/+	752.4E	10	106.7	160	1.48	43
Nonsevere	754.1E	8	67.9	83	1.30	27
	752.4D	10	101.3	89	0.88	45
				$\bar{x}(\pm SE)$	$1.21 \pm .57^{\alpha}$	38 ± 4
DI+/+	731.3I	11	113.8	79	0.69	0
Severe	731.3H	11	117.1	42	0.38	0
	735.1B	10	112.9	44	0.40	0
				$\bar{x}(\pm SE)$	$0.49 \pm .26^{\P}$	$0 \pm 0^{\dagger}$

Apical surface is the length in micrometers, measured along the apical surface in micrograph cross sections of principal cells; this measurement was an arbitrary standard to help assess vesicle density. † =significantly different from DI +/+ Nonsevere ($P<0.05$) and VII +/+ Normal ($P<0.025$). ¶ =significantly different from VII +/+ Normal ($P<0.001$). α =significantly different from VII +/+ Normal ($P=0.05$).

3.3 Cytoplasmic Vesicles

3.3.1 Quantitative Data -- The data concerning cytoplasmic vesicles are presented in Table 6. Qualitative analysis of representative micrographs from a DI +/+ Severe mouse (Fig. 14, p. 68) and a VII +/+ Normal mouse (Fig. 15, p. 68), shows the obvious difference in number of cytoplasmic vesicles[2] within one micrometer of the apical surface. ANOVA

[2]Because no statistically significant disequilibrium existed between the papillary and urinary osmolalities observed in the VII Os/+ mice (Fig. 5), the vesicles of this genotype were not examined in the present study. Since a difference in the proportion of responding cells for the CBA Os/+ compared to the VII +/+ Normal mice was observed (Table 2), additional studies are being performed to examine the cytoplasmic vesicles of the CBA Os/+ mice.

Figure 14. Transmission electron micrograph of the luminal region of a principal cell from a medullary collecting duct of a DI +/+ Severe mouse. Only rounded, non-coated vesicles within 1 micrometer of the apical surface were counted. x24,000.

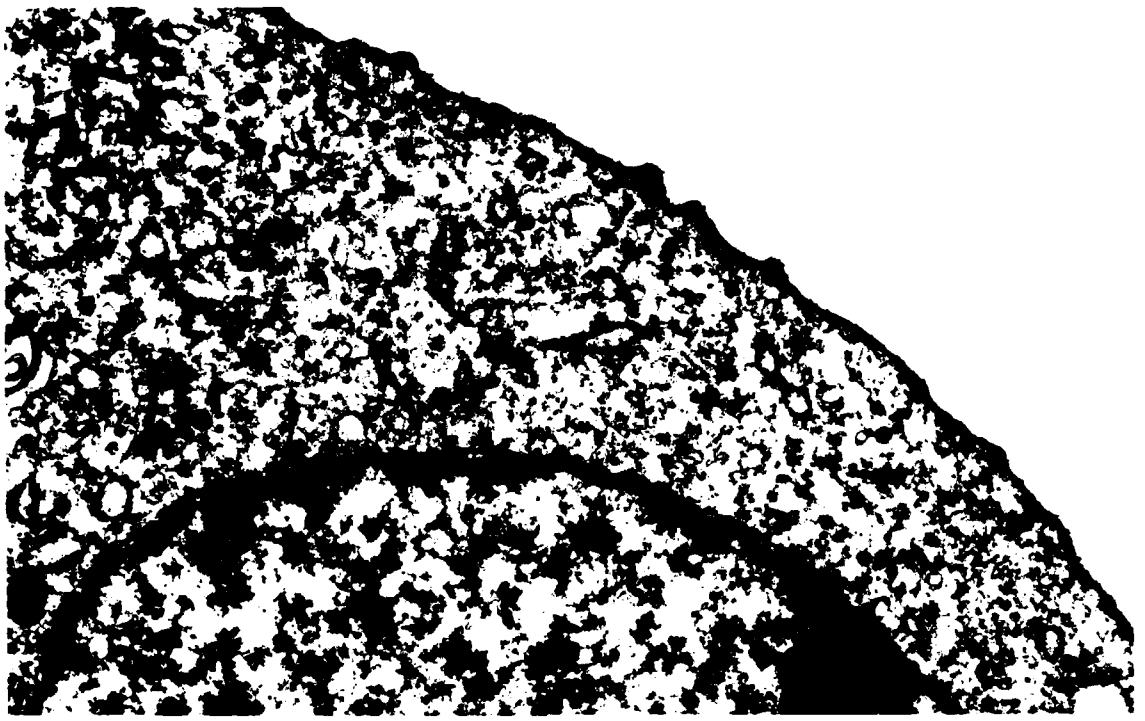
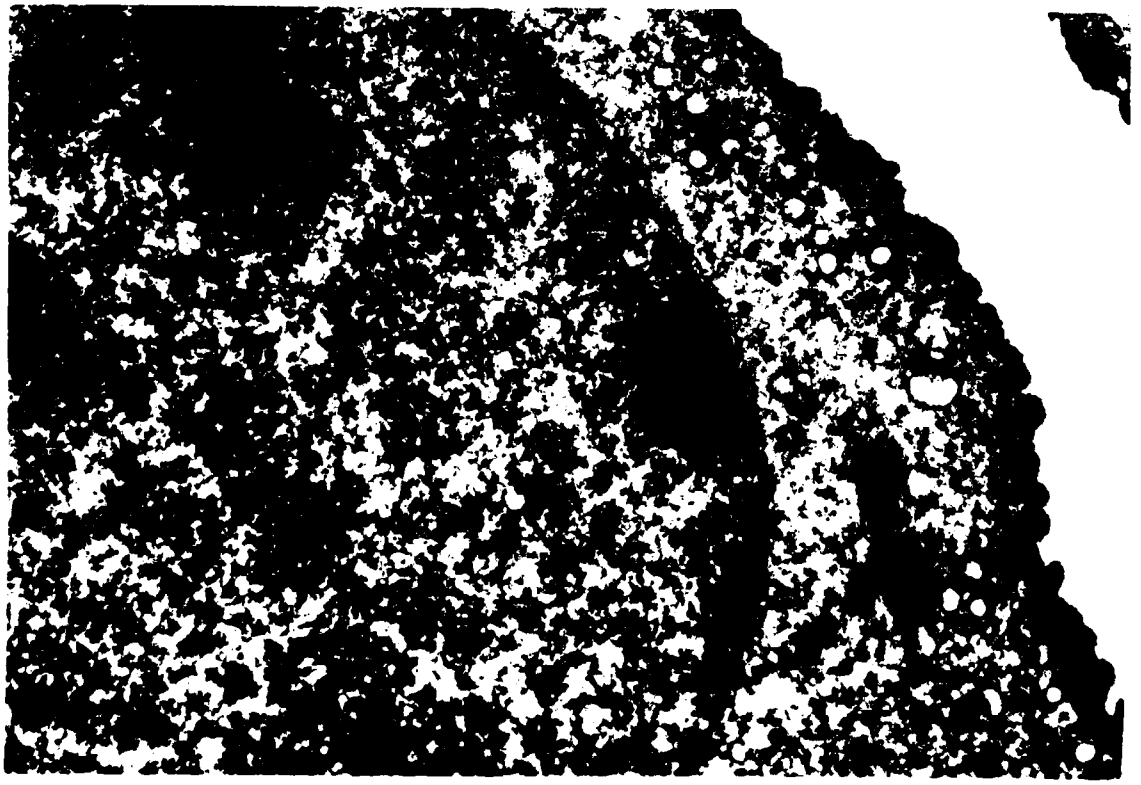


Figure 15. Transmission electron micrograph of the luminal region of a principal cell from a medullary collecting duct of a VII +/+ Normal mouse. Compare to Fig. 14. x24,000.



and Newman-Kuels Multiple Range Test revealed significant differences in this regard between DI +/+ Severe ($P<0.001$) and DI +/+ Nonsevere ($P=0.05$) from VII +/+ mice (penultimate column in Table 6). When comparing only three representative mice from each genotype, the Kruskal-Wallis Test for ANOVA by ranks and subsequent Multiple Comparison by the technique of Zar (175) showed that the mean IMP cluster frequency response of the DI +/+ Severe mice ($0\bar{+}0$)($n=3$) was statistically different from the mean response of the DI +/+ Nonsevere ($38\bar{+}4$)($P<0.05$) and VII +/+ Normal ($55\bar{+}7$) ($P<0.025$) mice. However, unlike the comparison of the IMP cluster frequency in all animals (Table 2, p. 60), the IMP cluster frequency for DI +/+ Nonsevere and VII +/+ Normal mice was not significantly different ($P=0.05$) because of the small sample size ($n=3$)(Table 6).

3.3.2 Cytoplasmic Vesicles and Frequency of IMP Clusters -- The data for IMP cluster frequency and number of vesicles per micrometer of apical surface in Table 6, were graphed and compared using Spearman's Rho Rank Correlation procedure (Fig. 16, p. 70). The computed value of Spearman's Rho, r_s, was 0.700. The critical value of Spearman's Rho for statistical tables for a "two-tailed" comparison was (r_s =0.700. Comparison of these two values would normally mean that [illegible] not enough information to say that there is signif[illegible] between the variables. However, given the fa[illegible] studies have shown only a positive corre[illegible] correlation is indicated in the grap[illegible] examine the relationship of thes[illegible] The critical value of [illegible] Therefore, IMP [illegible] m[illegible]

AD-A188 254 INTRAMEMBRANEOUS PARTICLE CLUSTER AND CYTOPLASMIC VESICLES IN MICE WITH N..(U) AIR FORCE INST OF TECH WRIGHT-PATTERSON AFB OH G I SHIELDS 1987 2/2

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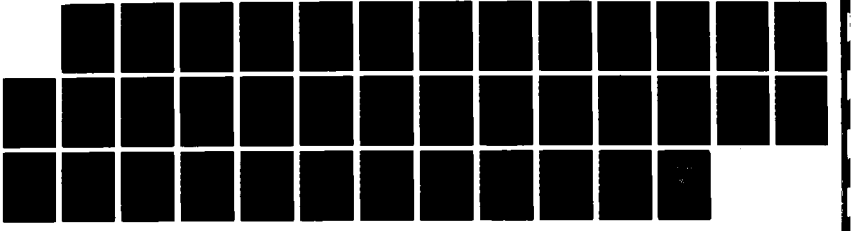


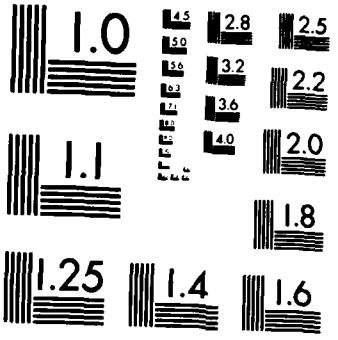
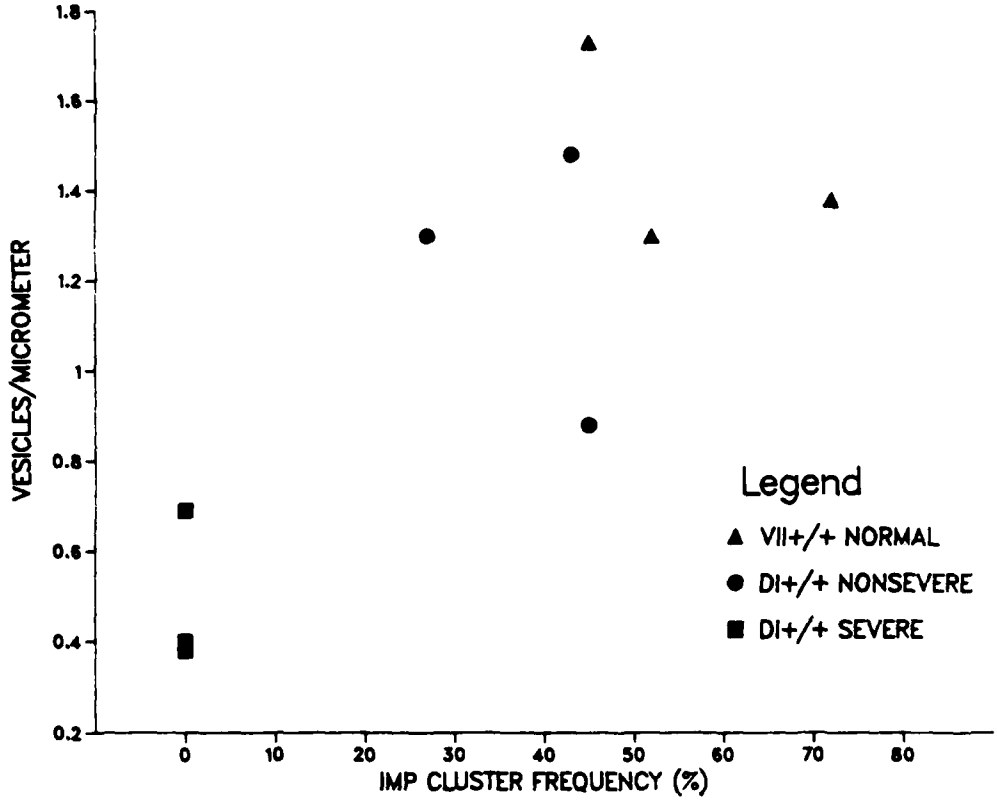


Figure 16. Relationship between cytoplasmic vesicles and IMP cluster frequency. Each symbol represents the mean number of vesicles observed per micrometer of apical surface examined versus % positive cells in an individual mouse. Spearman's Rank Correlation coefficient, r_s was 0.700 between the two variables, P=0.025.



4 DISCUSSION

The experimental results provide new information about all of the questions asked during this investigation. First, like the rat, but contrary to amphibians, increased water permeability in mice results from increased numbers of cells with IMP clusters rather than increased clusters in each cell. DI +/+ Severe mice, however, do not have IMP clusters, which is at least partly the cause of their lack of water permeability in the collecting duct system. Secondly, cytoplasmic vesicles were most abundant in principal cells of mice which had large numbers of IMP clusters, and less abundant in principal cells of mice which had few IMP clusters. And third, though the characteristics of the IMP cluster response were similar in control and DI mice, they were markedly different in mice with oligosyndactyly.

4.1 IMP Clusters

4.1.1 Frequency Response by Genotype -- No IMP clusters were found in the DI +/+ Severe mice in the present study. This response was analogous to the response previously observed in Brattleboro rats -- animals which do not have effective endogenous levels of vasopressin (165, 168), but which increase their urine osmolality and IMP clusters in parallel when given vasopressin (63,64). Recall that untreated Brattleboro rats produce copious amounts of dilute urine due to poor water permeability of the cells of the collecting ducts. They also have very few IMP clusters. Whether the DI +/+ Severe mice were examined untreated with their known elevated endogenous vasopressin levels (150), or after treatment with supranormal doses of DDAVP, none of the mice had IMP clusters, and all had very dilute urine ($\bar{x}$ U_{osm} = 180 mOsm/kg H_2O).

The similarity between the untreated Brattleboro rat and the DI +/+

Severe mouse is even closer if one takes into account Dennis Brown's recent re-analysis of the morphology of the principal cells of untreated homozygous Brattleboro rats: that the very small particle clusters observed in these rats may not be the "same" IMP clusters that are believed to be responsible for water permeability[1] (Brown, personal communication). In such a case, untreated, homozygous Brattleboro rats could have been described as having negligible or no, rather than minimal [$0.03 \pm .01$], IMP clusters per square micrometer of membrane (63,64), much like the DI +/+ Severe mice in the present study.

Both the VII +/+ Normal and the CBA Os/+ mice, which were concentrating their urine to high levels, had high frequencies of IMP clusters, 52 ± 5 and 84 ± 6 % positive cells, respectively. IMP cluster frequency and urinary concentrating ability in the Brattleboro rat study (64) had shown a similar relationship: that is, that IMP cluster frequency paralleled U_{osm} during the administration of exogenous vasopressin. In the acute situation in the Brattleboro rat, this response is probably the result of an increase in water permeability of the collecting duct system (168). The data of the present study suggest that this was also the case for the VII +/+ Normal and CBA Os/+ mice.

Recall that a significant disequilibrium between papillary and urinary osmolality (very likely due to defective water permeability of the collecting duct system) had been reported previously in the DI +/+ Severe mice (Fig. 6)(85). The same study also pointed out a sizeable, though statistically insignificant, disequilibrium between urinary and papillary osmolalities of the DI +/+ Nonsevere mice (85). The present

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[1] Harmanci et al (63) noted that these clusters were smaller and only "superficially resemble the much larger clusters of ADH-treated rats", but they counted these as IMP clusters to insure the scientific rigor of the study.

study shows that in addition to a large disequilibrium, the DI +/+ Severe mice had no IMP clusters, data which are in agreement with our assumption that IMP clusters reflect water permeability in the collecting duct system. Given that assumption, the IMP cluster frequency of DI +/+ Nonsevere mice should also be reduced, reflecting a partial disequilibrium. This was in fact the case, IMP cluster frequency being $33^{\pm}4$ %. These data provide further indirect evidence that IMP cluster frequency is related to the disequilibration observed in these mouse genotypes.

Statistical analysis of the data in Table 2 (p. 60) and Figure 12 (p. 63) demonstrates that a strong correlation [Spearman's Rank Correlation coefficient, $r_s = 0.891$, ($P<0.001$)], or association, exists between U_{osm} and the proportion of papillary collecting duct principal cells showing the IMP clustering response. When combined with the data from Kettyle and Valtin (85), this strong correlation, even within a given genotype, provides further indirect evidence of an obligate relationship between the IMP response and water permeability. Such a relationship has already been shown in anuran systems (18,74,75). In in vitro studies, Brown et al (18) and Kachadorian et al (74,75) using toad epidermis and urinary bladder, respectively, found increased osmotic water flow in proportion to increased IMP clusters. Thus, in all vasopressin-responsive epithelial cell systems examined so far, IMP clusters are greatly reduced or absent when water permeability is minimal, as indicated by either low U_{osm} as in the homozygous Brattleboro rat (63,64), or by negligible osmotic water flow as in the toad urinary bladder (74,75) or toad epidermis (18). Therefore, it is reasonable to suggest that the lack of IMP clusters in the DI +/+ Severe mouse as well as the reduced number of clusters in the DI +/+ Nonsevere mouse, are at least a major cause of the urinary concentrating defect that these

genotypes manifest. The comparison of the measured water permeability of isolated, perfused, collecting ducts and the status of the IMP clustering response of principal cells from the same ducts, could provide direct proof for such a role. These experiments are currently underway.

4.2 Characteristics of IMP Clusters

As previously mentioned, when examining the replicas of either VII +/+, DI +/+ Nonsevere, or CBA Os/+ mice for the presence of IMP clusters, it was often possible to find principal cells without the response adjacent to principal cells displaying a maximum response (Fig. 13, p. 64). This heterogeneity of response between cells led to the question of whether there might be a heterogeneity of particle response within each cluster, both within the same animal and between animals of different genotypes.

4.2.1 IMP Cluster Density -- In the two studies on the Brattleboro rat by Harmanci et al (63,64), a significant number of replicas from homozygous Brattleboro rats given exogenous vasopressin did not have any IMP clusters. Because they were thought to represent negative portions of cells which probably had IMP clusters, these data were averaged into the results, which were reported as frequency of IMP clusters per 100 square micrometers of membrane. It is possible that the cells without IMP clusters could truly have been nonresponsive cells (Harmanci, personal communication).

Examination of the data in the present study concerning the density of IMP clusters, expressed as clusters per square micrometer of apical surface (Table 3, p. 62), failed to reveal any differences between the three genotypes which had IMP clusters. VII +/+ Normal mice had 1.16 $\pm$0.07, CBA Os/+ animals had 1.08 $\pm$0.07, and DI +/+ Nonseveres had 1.13

$\pm$0.11 clusters per square micrometer. These data are in agreement with those of other studies (19,63,64).

Brown and Orci (19) reported a mean value of 1.2 clusters per square micrometer in male Wistar rats deprived of water for 24 hours. Harmanci et al (63) had reported a mean value of 1.17 $\pm$0.17 in collecting duct luminal membranes of vasopressin-treated homozygous Brattleboro rats. Additionally, data I have extracted from the results of a second study by Harmanci et al (64) show a similar value. Four homozygous Brattleboro rats treated with doses of vasopressin resulting in maximum U_{osm} had a mean cluster frequency of approximately 0.9 clusters per square micrometer of surface examined. The response of these four rats approximated a steady state condition. Less than maximum responses were, by experimental design, acute responses, and I did not include them in my calculations.

It must be noted that the previously mentioned data concerning proportion of cells responding with IMP clusters, were obtained from mice which were sacrificed during steady states of water diuresis and graded antidiuresis. It is possible, therefore, that during the transition phase between antidiuresis and various stages of water diuresis, a progression of IMP clusters per square micrometer of apical surface could occur. In fact, that response has been reported for toad urinary bladder during the onset of the hydrosmotic response to vasopressin (23,77). Further, very recent studies by Kachadorian in toad bladder reveal that in this epithelium the response under full vasopressin stimulation occurs in all cells, though the cells vary in the strength of the response (Kachadorian, personal communication). Nevertheless, in the study of Chevalier et al on toad bladder (23), the phenomenon of poorly responsive or nonresponsive cells adjacent to responsive ones was observed, even while the progression of IMP clusters per square micrometer usually found

during the acute response, was taking place.

An argument can be made concerning the validity of the IMP clustering response observed in the present study. Only in isolated instances was the entire apical surface of a principal cell exposed during the fortuitous fracturing process. Consequently, it is possible that some cells judged to be unresponsive, in fact could have contained IMP clusters in areas of the cell that were not exposed by fracture. Arguing against this possibility is the fact that all cells judged to be positive had IMP clusters distributed rather homogeneously over the entire surface exposed by the fracturing process. However, the data from the studies previously mentioned (19,63,64) when combined with the data from the present study, suggest that the steady state response of the mammalian collecting duct to vasopressin is, at least partially, one of cell recruitment.

4.2.2 Particles within IMP Clusters

4.2.2.1 Number of Particles per Cluster -- In order to check the mice for heterogeneity of response between genotypes, each IMP cluster which could be identified on the photograph of an E face replica was examined to determine how many individual particles comprised each cluster. Qualitative observations suggested that the CBA Os/+ animals would have more particles per cluster. In fact, two clusters which included over 100 particles were not included in the data, for fear that two fused clusters had been observed. Statistical analysis confirmed that although the mean number of particles in DI +/+ Nonsevere and VII +/+ Normal mice was not different, the mean for the CBA Os/+ mice was larger than both ($P<0.001$)(Table 5, p.66).

4.2.2.2 Size distribution -- The CBA Os/+ mice had many more clusters

containing a large number of IMPs than did the two other genotypes with clusters (Table 5, p. 66). The meaning of this difference in distribution and the difference in the number of particles per cluster is at present unknown.

One possible clue might lie in the fact that the etiology of renal disease in the DI +/+ Nonsevere mice is different from that of the CBA Os/+ mice. The Os/+ mice are in chronic renal failure due to a dearth of nephrons. One could speculate that the CBA Os/+ mice may be volume depleted and therefore have very high endogenous vasopressin levels, to which their principal cells are perfectly capable of responding. Therefore, they would be in a state of high response with clusters, without the correct osmotic water flow across the principal cell luminal membranes acting as "feedback" to modulate the clustering response. This "feedback" action has been postulated before by Masur (100) and Kachadorian (109). In contrast, the DI +/+ Nonsevere and VII +/+ Normal animals may have the clustering response properly modulated by osmotic water flow across the apical membrane and therefore not have IMP clusters in excess of the amount "specified" by a given level of vasopressin. This same interpretation could be applied to help explain why the CBA Os/+ animals have a larger percentage of responding cells but a lower U_{osm} than VII +/+ Normal mice[2].

4.3 Cytoplasmic Vesicles

The results of the present experiments also provide important data

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[2]Two facts must be kept in mind. No data are currently available on whether any osmotic disequilibrium between urinary and papillary osmolalities exists in this genotype. However, no statistically significant disequilibrium was found in a similar genotype, VII Os/+, in the study of Kettyle and Valtin (Fig. 6, p. 32)(85). With a smaller kidney and shortened corticopapillary gradient, urinary concentration even at full collecting duct water permeability cannot be as great as in VII +/+ Normal mice.

on the second question of this investigation. A comparison of the luminal regions of principal cells from DI +/+ Severe and VII +/+ Normal mice (Figs. 14 and 15, p. 68), demonstrates a qualitative difference between the two genotypes in numbers of cytoplasmic vesicles. The vesicles appear to be much more numerous in the VII +/+ Normal mice, which also have numerous IMP clusters and no defect in urinary concentration. In addition, the data in Table 6, p. 67, and Figure 16, p. 70 show that a strong correlation [Spearman's Rank Correlation coefficient, r_s=0.70 (P=0.025)] exists between the number of IMP clusters per square micrometer and the number of vesicles per micrometer of membrane within one micrometer of the apical surface. These data suggest that the frequency of IMP clusters in principal cells of mouse collecting ducts is related to the number of cytoplasmic vesicles in those cells.

Data from the first part of the present investigation have reinforced the suggestion of Kettyle and Valtin that DI +/+ Severe mice have a severe defect in the water permeability of the collecting duct system, and that DI +/+ Nonsevere mice might have a similar but milder defect. The differences in number of cytoplasmic vesicles, which were observed between the three genotypes, closely parallel the differences in frequency of IMP clusters in the same genotypes. The apparent interrelationship of IMP cluster frequency, numbers of cytoplasmic vesicles, and U_{osm} seen in these genotypes suggests that it might be reasonable to expect these cellular phenomena to exist in an obligate relationship.

The original idea behind examining these cells for the presence of cytoplasmic vesicles was that the vesicles might be subserving a function in mouse collecting duct principal cells similar to that of the tubular vesicles (aggrephores) in toad bladder granular cells. If so, comparisons might be drawn between the two systems. While the present study has

not determined the actual function of the vesicles in mice, there was, nevertheless, a correlation between the proportion of cells which respond with IMPs and the number of vesicles which the cells contained (Fig. 16). Therefore, a case can be made, which proposes that a function similar to that in toad bladder exists for the cytoplasmic vesicles in the collecting ducts of mice.

Recall that Muller et al had demonstrated a linear correlation between vesicle fusion events and IMP cluster response in toad bladder (108). Based on the assumption that IMP clustering is an accurate measure of water permeability in the mouse collecting duct, and the possibility that these vesicles are the source of the IMP clusters (as they appear to be in the toad bladder), one could speculate that a lack of the vesicles in mice would be accompanied by a lack of IMP clusters, and hence by reduced water permeability.

But, there are other possible explanations for the physiologic consequences of a lack of these cytoplasmic vesicles. Since the thin sections used for quantification of vesicles could not be utilized simultaneously for freeze fracture analysis, it is possible that some of the vesicle counts were not matched appropriately with their simultaneous response of IMP clusters. It is, therefore, possible that data pertaining to the number of vesicles include counts from cells which respond with clusters as well as cells which do not so respond. It is more likely, however, that the random nature of the selection process of cells to be studied has minimized any potential between-class errors due to the above-mentioned mismatch, so that any differences which have been statistically determined are actual.

Alternatively, the phenomenon of decreased numbers of cytoplasmic vesicles may be a pathophysiological defect separate from decreased U_{osm}. Arguing against this interpretation, however, is the fact that both the

DI +/+ Severe and Nonsevere genotypes can concentrate their urine to a greater degree than that observed at the time of the experiments, namely, during the period from shortly after birth until the onset of the more severe disease at approximately six months (Fig. 5)(167). It is possible that the defects in vesicles and urinary concentration are unrelated, but still occur together. It is more probable, however, that these two defects are related, since the severity of expression of the vesicle defect seems to be in direct proportion to the number of IMP clusters in collecting duct cells. To my knowledge, no studies have been done to ascertain whether there is a progression with age of DI +/+ Severe and DI +/+ Nonsevere mice, in either the number of cytoplasmic vesicles or the ability to produce or insert intramembranous particle clusters, irrespective of vasopressin stimulation. Such a study might determine whether loss of ability to concentrate urine occurs over the same time course (in months) as the decrease in IMP clustering and the decrease in vesicle number. And, such a finding would more inextricably associate the three phenomena.

There are still other explanations that can be offered for the effects which accrue to principal cells from the lack of cytoplasmic vesicles. Unfortunately, the lack of data concerning the cellular mechanisms of mammalian principal cells allows only speculation in this regard. Since there currently is no morphological marker to help delineate whether the IMPs sometimes observed in the membranes of the vesicles are preformed IMP clusters, such as is the case in toad bladder (11,12,42,69,171), it is not known whether these vesicles are destined to be exocytosed, or, in fact, have recently been endocytosed. There are vesicles in collecting duct principal cells of mice which have a bristle coat and which are therefore, likely to be endocytotic in nature (Brown, unpublished observations). In contrast, the tubular vesicles observed in

toad bladder, which are thought to be involved in the shuttling or recycling of IMPs (172), do not have bristle coats. Yet the preponderance of evidence suggests that these vesicles without a bristle coat are both endocytosed and exocytosed (57,99,108,172,173). Several studies suggest that this retrieval, or endocytotic, mechanism occurs in toad bladder in response to a decrease or washout of vasopressin (77,100,108,172,173), even though this mechanism does not involve coated pits. Additionally, osmotic water flow, such as would occur during stimulation with vasopressin in vivo, apparently partially inhibits the exocytosis of the tubular vesicles into the apical membrane (109).

Therefore, there are several questions about the lack of cytoplasmic vesicles in collecting ducts of DI +/+ Severe and Nonsevere mice, which this investigation has left unanswered. Would a quantification of cytoplasmic vesicles of principal cells from CBA Os/+ mice, which have the highest proportion of responding cells yet observed, yield a value greater than that of the VII +/+ Normal mice? Are the vesicles exocytotic or endocytotic in nature? The initial assumption of the present study concerning the nature of these vesicles was that the vesicles consisted of membrane which had been recently endocytosed and had its bristle coat removed. (Some indirect evidence from pilot experiments suggests that the latter is probably the case [see APPENDIX]. The IMP clustering response was induced in DI +/+ Severe mice during in vitro incubations with appropriate agonists. The response was not unlike the IMP clustering observed in VII +/+ Normal mice. However, there were comparatively few cytoplasmic vesicles observed in untreated DI +/+ Severe mice. If the vesicles were primarily exocytotic in nature, there almost certainly were not enough already in the cytoplasm to serve as the source of the IMP clusters observed in the incubated DI +/+ Severe tissues.)

Currently experiments are underway to try to directly relate the IMP cluster response and collecting duct water permeability. These experiments entail the direct measurement of water permeability by perfusion of isolated tubules and a subsequent freeze fracture analysis of principal cells from the same tubules. Data from these experiments should provide direct evidence, either in support of, or against, a causal role for IMP clusters in mediating the increase in water permeability in the mouse collecting duct system in response to stimulation by neurohypophysial hormones.

The present study has provided important data on the IMP clustering response in principal cells of the mouse collecting duct system, both in health and in defects of urinary concentration of several origins. The characterization of genotypic variation in the number of cytoplasmic vesicles is an important first step in understanding, not only the mechanism which controls luminal membrane permeability in the mouse collecting duct system, but also the mechanism, or mechanisms, operative in other epithelia that respond to stimulation with neurohypophysial hormones. In addition, the present study has materially added to the existing indirect evidence which suggests that the clustering of IMPs plays an obligate role in mediating the increase of water permeabilty in the mouse collecting duct system in response to neurohypophysial hormones.

4.4 Summary of Results

4.4.1 IMP Clusters in DI Mice -- No clusters were found in DI +/+ Severe mice. VII +/+ Normal and CBA Os/+ mice had high frequencies of IMP clusters, while DI +/+ Nonsevere mice had intermediate frequencies. These data are in close agreement with the study of Kettyle and Valtin (85), which reported on the basis of more indirect evidence, no defect of

water permeability in VII +/+ Normal and VII Os/+ mice (analogous to CBA Os/+ in the present study); defective water permeability in DI +/+ Severe mice; and a possible intermediate defect in DI +/+ Nonsevere mice. In addition, the IMP cluster data from all mouse strains, when compared statistically with urine osmolality, provide further indirect evidence that, in mice as in anurans, IMP clusters mediate, at least in part, the water permeability of epithelia that respond to vasopressin.

4.4.2 Characteristics of IMP Clusters -- While there was no difference in mean IMP clusters per micrometer of apical surface between any of the mouse strains that showed the clustering response, CBA Os/+ mice did have more IMPs per cluster and more of the larger clusters than either DI +/+ Nonsevere or VII +/+ Normal mice. The significance of this difference is not known at this time. However, the etiology for the nephrogenic defect of urinary concentration in the CBA Os/+ mice is different from that found in DI +/+ Nonsevere mice, and the IMP clustering response may, after further study, help to explain this difference.

4.4.3 Cytoplasmic Vesicles -- It is not known whether cytoplasmic vesicles in the principal cells of the collecting duct system of mice are the source of IMP clusters. In the present study there was a very strong correlation between frequency of IMP clusters and the presence of cytoplasmic vesicles (within 1 micrometer of the apical surface). No causality can be inferred between these two morphological characteristics since they were examined as independent variables. However, given the data from Brown and Orci, which suggest that in the rat IMP clusters are coincident with coated pits (19), it would not be unreasonable to hypothesize that cytoplasmic vesicles in mice subserve a function similar

to aggrephores in the toad bladder. That is, the cytoplasmic vesicles may well be the recyclable source of IMP clusters.

5 APPENDIX

5.1 Introduction

As previously mentioned, it was discovered early in the present study that the DI +/+ Severe mice, after the onset of florid symptoms, did not have any IMP clusters despite treatment with exogenous vasopressin, even though they had been able to concentrate their urine at an earlier age. Jackson et al had shown that these animals with nephrogenic diabetes insipidus had elevated levels of cAMP phosphodiesterase (70). Subsequent experiments were conducted by Kiebzak, Holets, Kusano and Dousa in the same laboratory which they attempted to correct the concentrating defect by reversing the high activity of cAMP phosphodiesterase through the in vivo administration of 1-methyl-3-isobutyl xanthine (MIX), an inhibitor of cAMP phosphodiesterase. In fact, the DI +/+ Severe animals given MIX were able to concentrate their urine somewhat (86). However, due to the ubiquitous nature of cAMP-mediated mechanisms, these animals also looked ill and lost weight. The study, therefore, could not rule out the possibility that U_{osm} had increased because of a decrease in glomerular filtration rate (86). Nor could it rule out that the increase in U_{osm} was due to effects of some other, non-renal system, whose operation is mediated by cAMP. Nevertheless, the idea of attempting to correct the defect was appealing.

5.2 Rationale of Approach

With that idea in mind, two basic strategies were employed in pilot studies designed to probe whether the lack of IMP clusters in DI +/+ Severe mice could be reversed in vitro. MIX would be used to attempt to increase intracellular cAMP by preventing its premature breakdown due to

elevated cAMP phosphodiesterase activity. Also, attacking the cAMP problem from a different approach, forskolin would be used to attempt to produce "extra" intracellular cAMP and saturate the high cAMP phosphodiesterase activity. The "extra" cAMP would result because forskolin has been shown to be an activator of adenylate cyclase, and in several systems, to increase the amount of cAMP produced by equal amounts of receptor agonist (138,140). In fact, forskolin increased osmotic water permeability in the isolated, perfused, cortical collecting tubule of rabbits (30), presumably by means of a cAMP-mediated mechanism. The in vitro method to test these two strategies, chosen for its simplicity, was that of incubating freshly-excised, papillary tissue slices in culture medium with various combinations of agonist and inhibitor and examine the slices for the presence of IMP clusters.

5.3 Initial Experiments

5.3.1 Materials and Methods -- Papillae from DI +/+ Severe mice were excised using the same procedures described in the section on Materials and Methods. The papillae were put immediately into Dulbecco's Modified Eagle Medium (DMEM)(Gibco Laboratories, Chagrin, OH) and maintained at 10 to 14°C. They were cut to 50 micrometer thick sections on an Oxford 501 vibratome, using procedures identical to those described in the Freeze Fracture section of Materials and Methods, with one exception. The buffer well of the vibratome was fitted with a smaller plastic well which was manually filled with a mixture of alcohol and dry ice to cool the buffer to between 10° and 14°C. With the softer, unfixed papillae, it was necessary to grasp the sections with a fine forceps as they were cut from the papillary block of tissue, or the slice would wrinkle and tear. The separate sections were stored in a container of cold, oxygenated DMEM until all papillae were sectioned, approximately 1.5 hours. Groups of

five to ten sections were then incubated in medium with the various agonists and inhibitors for 30 minutes at 37°C in 96 well cluster plates, bubbled with 95% O_2 and 5% CO_2. Sections from nine DI +/+ Severe mice (average U_{osm} = 110 mOsm/kg H_2O) were pooled and randomly assigned to different treatments. The combinations of agonists and/or inhibitors dissolved in DMEM included: fixing slices immediately; incubating slices in DMEM only; 10^{-5}M forskolin with 10^{-4}M vasopressin; 5 x 10^{-4}M MIX; and 10^{-5}M DDAVP (1-desamino-8-D-arginine vasopressin) with 5 x 10^{-4}M MIX. At the conclusion of the incubation period, the sections were put into 2% glutaraldehyde/sodium cacodylate buffer for 2 hours, coded, and sent to Geneva for freeze fracture analysis, utilizing the methods previously described.

5.3.2 Results -- IMP clusters could not be found in replicas from either of the control treatments, as had been the case with the 16 DI +/+ Severe mice previously examined. Replicas from the forskolin-vasopressin slices had 4 out of 7 cells positive for IMP clusters (57%). Replicas from the MIX-treated sections exhibited a similar response: 17 out of 27 cells positive (63%). Paradoxically, replicas from the DDAVP-MIX treatment, a strategy which should also result in increased intracellular cAMP, failed to show the clustering response: 0 out of 7 cells (0%). Replicas from apical membranes of a much greater number of cells that were examined during this experiment showed severe membrane damage and therefore could not be scored.

5.3.3 Discussion -- There are several possible explanations for the failure of some treatments to induce the formation of IMP clusters. For example, since the actual intracellular concentration of cAMP was never assayed in the present study, it is possible that some of the agonists

did not penetrate well into the tissue. Additionally, while DDAVP has been shown in whole tissue preparations to stimulate adenylate cyclase, it has not been used in vitro to directly stimulate a measurable increase in intracellular cAMP (Thomas Dousa, personal communication). Without directly measuring cAMP content, it is not possible to ascertain whether DDAVP actually increased cAMP content. On the other hand, DDAVP has been widely used as a replacement for vasopressin. In numerous experiments DDAVP has been able to replace that action of vasopressin which results in the concentration of urine. However, in recent studies by Kusano et al (91), defective cAMP accumulation in isolated tubules of DI +/+ Severe mice was only fully restored when stimulated by treatment with a combination of MIX, vasopressin, and forskolin. This combination was not tried during the pilot experiments of the present study.

The fact that some of the membranes were damaged may also cast doubt upon the validity of the results of these pilot experiments. For example, incubation in the cold has been shown, in at least one model system, to cause the aggregation of IMP particles (147). However, this type of aggregation may be easily distinguished from true IMP clustering in both the toad bladder (79,108) and principal cells of mouse papillary collecting ducts (Brown, unpublished observations). Numerical and spatial organization is visible within the true IMP clusters, which is absent in the aggregation response due to cold or other damage. Moderate cold, 10.5 $\pm$0.4°C, well within the range used in the present study (10 to 14°C), did not cause aggregation of IMPs in experiments on toad bladder (79). On the contrary, it inhibited vasopressin-stimulated formation of IMP clusters by 51.5 $\pm$7.2% (79). Likewise, in a more recent study, Riddle, using unkeratinized, human, fetal epidermis, showed no difference in IMP clustering in tissues incubated in the cold (25 to 30°C) versus tissues incubated at body temperature (37°C)(129). Since those previous

experiments revealed no identifiable pattern of aggregation response to temperature variations, it appeared more likely that some other physical condition or conditions, such as the trauma of slicing, or hypoxia/anoxia of the tissue, was the cause of the membrane damage in our experiments.

5.4 Modified Experiments

5.4.1 Materials and Methods -- In an attempt to reduce membrane damage, additional experiments were performed using sections from individual, rather than pooled, papillae, in the hope that less delay in processing would minimize or prevent damage. This new procedure reduced the time from excision of the papillae from the animal until introduction into an incubation well from 1.5 hours to less than 1 hour. In addition, the treatments of agonist and inhibitor were changed slightly. The new treatments, applied in addition to those listed above, included: MIX, from 5×10^{-2}M to 5×10^{-9}M; vasopressin, from 10^{-4}M to 10^{-10}M; the same concentrations of vasopressin with 5×10^{-4}M MIX; and 8-para-chlorophenylthio cyclic AMP (super cAMP)[a more fat-soluble analogue of cAMP] at 10^{-3}M and 10^{-6}M. The results were disappointing.

5.4.2 Results -- The condition of the tissues in general was vastly improved, although a number of membranes were still damaged. Unfortunately, true IMP clusters were found on most replicas, regardless of treatment, including the control animals incubated only in DMEM. The experiment was repeated.

5.5 Final Experiments

5.5.1 Materials and Methods -- For this last attempt, the cooling mechanism of the vibratome was modified, as were the concentrations of agonist and inhibitor. Two previous investigations, that of Parisi et al

(119) and that of Pinto Da Silva (125), had shown that changes in pH could affect intramembranous particles, though these studies had been concerned with frog bladder and human erythrocytes, respectively. The possibility existed in the present experiments that CO_2 vapors from the dry ice might have dissolved in the buffer solution, lowered the pH of the vibratome buffer, and caused aggregation of particles in the membranes. Therefore, the vibratome was fitted with a copper coil through which was circulated a mixture of antifreeze and water, which itself was cooled by circulation through a separate thermos containing a dry ice and water slurry. Also, incubation treatments were again changed to include: MIX at 10^{-6}M; vasopressin at 10^{-4}M; and super cAMP at 10^{-3}M. Familiarity with required techniques, and sectioning only one papilla at a time before incubation, reduced the time from removal of the kidneys from the animal until the beginning of incubation to less than 30 minutes. Once again, however, the results left much to be desired.

5.5.2 Results -- The condition of the membranes was now improved to the point that these last replicas were nearly as good as replicas from tissue fixed immediately after excision. However, there were still no differences between the numbers of IMP clusters observed in control animals and those observed in experimental animals, with all treatments having apparently equal effects.

5.6 Outlook

The ambiguous results from these pilot experiments are not without some merit. Unfortunately, the small number of cells that produced IMP clusters in response to stimulation by neurohypophysial hormones or agonists certainly makes the drawing of conclusions difficult. However, recall that no IMP clusters had been found in the papillae of 16 previous

DI +/+ Severe mice, which were fixed either immediately after excision, or after 30 minutes of incubation in DMEM (Table 2). A most important finding, therefore, is that DI +/+ Severe mice apparently have the biochemical and/or biophysical cellular mechanisms required for an IMP clustering response, since clustering was induced by various experimental manipulations. That fact was evident throughout the whole series of pilot experiments. Whether this IMP clustering response is connected with water permeability has not been proven, but the morphology of the induced IMP clusters is identical to that of clusters from both DI +/+ Nonsevere and VII +/+ mice.

There is one ready, albeit speculative, explanation for the observation of IMP clusters in DI +/+ Severe mice after in vitro incubation, for it has experimental precedent. Brown et al (16,17) found that they could cause exocytosis of 50% of the granules available in unstimulated toad bladder granular cells simply by stretching the bladder for as little as 5 minutes. Stretched bladders also had significantly increased IMP clusters in their apical membranes. Minsky and Chlapowski (102) also reported increases from 85-94% in the membrane surface area due to stretch in urinary bladders from several mammals. The bladders of guinea pigs, gerbils, hamsters, rabbits, and rats responded to hydraulic stretch with increases of membrane surface area, which the investigators attributed to fusion of cytoplasmic, discoidal vesicles with the bladder membrane surface (102). Interestingly, they also found a correlation between tonicity of the urine of each species and the density of discoidal vesicles present in the cytoplasm (102). Data reported by Lewis and de Moura on similar experiments with rabbit urinary bladder (95) were in general agreement with the data of Minski and Chlapowski (102). Lewis and de Moura stretched bladders by imposing a water pressure gradient (which they termed a "punch") on the bladders.

These "punches" caused an average increase of 22% in apical membrane surface area. The investigators attributed the increase to the addition of membrane from the fusion of cytoplasmic vesicles. Capacitance measurements had also been used to rule out the possibility that simple stretching of pre-existing folds in the membrane could be the cause of such an increase (95).

It is possible -- perhaps probable -- that in the present study the process of sectioning the live papillae involved sufficient stretching to have caused the exocytosis of preexisting cytoplasmic vesicles which contained preformed IMP clusters. Thus, in the first pilot study, the 1.5 hours which elapsed may have provided sufficient time for endocytosis to follow the "mechanical exocytosis", so that no IMP clusters were observed in controls. But such a process would still allow for IMPs to be reintroduced with appropriate stimuli (forskolin-vasopressin or MIX). Unfortunately, the same processing delay also probably accounted for either tissue hypoxia or anoxia, and ultimately, disturbed membranes. In the subsequent studies, the more rapid processing certainly improved the condition of the cells and/or membranes, but also possibly did not allow enough time for any IMP clusters introduced into the membrane by stretching to be recycled by endocytosis. Irrespective of these processing difficulties, the data do demonstrate that, at least in vitro, DI +/+ Severe mice do have the capability of forming IMP clusters. This fact alone tends to add credence to the theory that DI +/+ Severe mice have a defect in water permeability, which is caused at least partially by a defect somewhere in the second messenger cascade between the generation of cAMP and the insertion of IMP clusters.

Jackson and colleagues have investigated cAMP metabolism in this cascade. In addition to the increased activity of cAMP phosphodiesterase in collecting ducts of DI +/+ Severe mice, they reported that in vitro

production of vasopressin-stimulated cAMP in medullary collecting ducts was lower than in normal mice (70). Further, an even lower adenylate cyclase activity was found in medullary thick ascending limbs of Henle (mTALH) in these mice, which could contribute to a deficient reabsorption of NaCl from those limbs. Therefore, at least part of the difficulty that these animals have in concentrating their urine might stem from an inability to build up a sufficient corticopapillary gradient. Further studies will be required to assess the relative contribution of each of these phenomena toward the entire defect in urinary concentrating ability.

When combined with data from previous studies, the information gained from the present investigation supports the notion that elevated activity of cAMP phosphodiesterase in medullary collecting ducts of DI +/+ Severe mice plays a role in the inability of these mice to concentrate their urine. Experiments currently underway in this laboratory which will assess directly, collecting duct water permeability and the coincident IMP clustering response, may shed further light on this defect and lead to a better understanding of the mechanisms that alter water permeability in the principal cells of mammalian collecting ducts.

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